Association of Alzheimer's Disease Progression with White Matter Hyperintensities and Blood Biomarkers

Mikula, Margit

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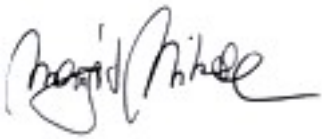

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“Du sollst Dich nicht nach einer vollkommenen Lehre sehnen, sondern nach einer Vervollkommung Deiner selbst” [Hermann Hesse]

“Space and time are the framework within which the mind is constrained to construct its experience of reality” [Immanuel Kant]

“Every truth passes through three stages before it is recognized. First, it is ridiculed. Second, it is opposed. Third, it is regarded as self-evident.” [Arthur Schopenhauer]

Abstract

Alzheimer's disease (AD) is the most common form of dementia affecting around 70% of dementia cases. The global prevalence of dementia is estimated to be 44.4 million people. The pathological hallmarks of AD are amyloid plaques and neurofibrillary tangles. AD impairs many cognitive functions including memory, language, abstract thinking, and spatial orientation and alters mood and behaviour. These symptoms become gradually more severe, affecting common daily activities until eventually full time care is required. There is currently no cure. A large number of promising drugs are being tested in clinical trials, but their effect on disease progression is difficult to assess using existing psychometric measures. New biomarkers, including cognitive-, MRI-, and blood-based measures could be combined to accurately predict disease progression.

First, potential associations of 1-year cognitive decline with baseline volumes of MRI White Matter Hyperintensities (WMH) from the whole brain, were evaluated. Higher baseline WMH volumes were found to be significantly associated with greater AD-cognitive decline (number of subjects (n) =84, $P<0.05$), with a median, yearly, cognitive change of -1.2 points in the Minimal-Mental-State-Exam, 1.5 points in ADAS-cog, and 1.3 points in Clinical-Dementia-Rating.

Second, WMH volume was associated with an identified panel of mRNAs (trainingset: n=53, $P<0.001$, $R^2=0.75$; validationset: n=21, $R^2=0.11$), and with proteins (trainingset: n=59, $P<0.001$, $R^2=0.76$; validationset: n=30, $R^2=0.03$), in individuals with AD. Disease progression, measured by AD-cognitive decline in CDR-SOB points, was associated with an identified panel of mRNA transcripts (trainingset: n=46, $P<0.001$, $R^2=0.86$; validationset: n=16, $R^2=0.13$).

It can be suggested that WMH and cognitive disease progression affect mRNA expression and protein concentration in blood. A limitation was that there were only small independent validationsets. Future work could investigate the identified molecular candidates in larger subject groups, and if confirmed, they could contribute to defining a blood-based disease progression biomarker panel.

Key Publications

Conference presentation

Mikula M., Sattlecker M., Proitsi P., O'Sullivan M., Simmons A., Mecocci P., Soininen H., Tsolaki M., Vellas B., Lovestone S., and Hodges A.
"Association of MRI White Matter Lesions and Cognitive Decline in Alzheimer's Disease" presented in section Diagnosis and Prognosis: White Matter and Cognition
Alzheimer's Association International Conference 2013, Boston, Massachusetts

There were four recent publications related to this work using the same cohorts (AddNeuroMed and Clinical Dementia Register), and partially similar methods such as linear mixed models and WEKA software:

1. Lunnon K., Sattlecker M., Furney S., Coppola G., Simmons A., Proitsi P., Lupton M. K., Lourdusamy A., Johnston C., Soininen H., Kłoszewska I., Mecocci P., Tsolaki M., Vellas B., Geschwind D., Lovestone S., Dobson R., and Hodges A.
"A blood gene expression marker of early Alzheimer's disease. "
Journal of Alzheimer's disease 2013

2. Hye A., Riddoch-Contreras J., Baird A., Ashton N., Bazenet C., Leung R., Westman E., Simmons A., Dobson R., Sattlecker M., Lupton M., Lunnon K., Keohane A., Ward M., Pike I., Zucht H. D., Pepin D., Zheng W., Tunnicliffe A., Richardson, J., Gauthier S., Soininen H., Kłoszewska I., Mecocci P., Tsolaki M., Vellas B., and Lovestone S.
"Plasma proteins predict conversion to dementia from prodromal disease"
Alzheimer's & Dementia 2014

3. Kiddle S. J., Sattlecker M., Proitsi P., Simmons A., Westman E., Bazenet C., Nelson S.K., Williams S., Hodges A.,Johnston C., Soininen H., Kłoszewska I., Mecocci P., Tsolaki M., Vellas B., Newhouse S., Lovestone S., and Dobson R.
"Candidate blood proteome markers of Alzheimer's disease onset and progression: a systematic review and replication study."
Journal of Alzheimer's disease 2014

4. Sattlecker M., Kiddle S.,Newhouse, S.,Proitsi P., Nelson S., Williams S., Johnston C., Killick R., Simmons A., Westman E., Hodges A., Soininen H., Kłoszewska I., Mecocci P., Tsolaki M., Vellas B., Lovestone S., and Dobson R.
"Alzheimer's disease biomarker discovery using SOMAscan multiplexed protein technology"
Alzheimer's & dementia: the journal of the Alzheimer's Association 2014

Organisation of the thesis

PART I – State of the art: provides essential background knowledge of AD, and the subsequent analysis.

PART II – Data and experiments: there are two main studies.

PART III – A wider horizon for future disease related scenarios is drawn.

Table of Contents

Index of Tables

List of Figures

List of Abbreviations

PART I: STATE OF THE ART

Chapter 1. Alzheimer's disease and its manifestations

1 Epidemiological facts and figures

Alzheimer's disease (AD), the most common form of dementia, is an enormous global challenge for societies and their health care systems which have to be adapted by improving the quality of care while containing costs. The implementation of appropriate policies is a matter of current worldwide endeavour. To date, over 44 million people globally are affected by dementias and new cases are expected to double by 2030 and more than triple by 2050 to 115 million (Prince et al. 2013). In the European Union, population-based studies indicate that the age-standardized prevalence in people older than 65 is 4.4 % for AD and 6.4 % including other forms of dementia; the prevalence of dementia increases with age rising up to 28.5% at age 90 years and older (Lobo et al. 2000). In the United Kingdom alone, 800 000 people are currently affected by dementia, a figure which might increase by 160 000 per year (Alzheimer's Research UK 2013). Disturbingly, the numbers in low- and middle- income countries are vastly increasing, potentially accounting for 70% of worldwide dementia cases by 2050 (Prince et al. 2013).

2 Clinical Diagnosis

Alzheimer's disease (Alzheimer 1907; Glenner 1981) is an irreversible, progressive neurodegenerative disorder characterised by a variety of cognitive deficits. AD usually begins with memory decline, affects behaviour and personality (Bonate & Howard 2011), and eventually full time care is required. In the past, AD patients have often been diagnosed only when showing behaviour symptoms such as aggression, dis-inhibition, and agitation and have been institutionalised only when they presented a risk for themselves and other people (Eastley & Wilcock 1997). In recent decades, established criteria have been described to facilitate and standardise the diagnosis of Alzheimer's disease in clinical and research context. The diagnosis persists under the paradigm of diagnosing "Probable Alzheimer's disease" referring to the fact that only after histopathological examination the presence of AD is 100% confirmed. For clinical diagnosis, the National Institute of Neurological and Communicative Disease and Stroke and Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al. 1984; McKhann et al. 2011) and the Diagnostic and Statistical Manual of Mental Disorders 5th edition (DSM-V) (Arlington 2013) specify eight cognitive domains that may be impaired in AD: memory, language, perceptual skills, attention, constructive abilities, orientation, problem solving and functional abilities. The minimal criteria for assessing cognitive or behavioural impairment involve at least two of the three domains: (i) acquisition and retention of new information; (ii) reasoning and handling of complex tasks; (iii) visuo-spatial abilities and language. Additionally, insidious onset, known history of worsening cognition, learning and recall of recently

learned information, word-finding, face recognition, impaired reasoning, judgement, and problem solving, are also taken into account. For accurate diagnosis of AD various co-morbidities should be excluded such as other types of dementia, diabetes, depression, severe infections, schizophrenia, or vitamin B deficiency. Other types of dementia include Dementia with Lewy bodies (DLB), Vascular Dementia (VD), Fronto-Temporal Dementia (FTD), and other less common forms. While most of dementias overlap with AD certain symptoms make them sufficiently distinct:

(1) DLB is usually accompanied by a progressive decline in mental abilities and the formation of lewy bodies (proteins) in the brain (Fujishiro et al. 2013). In DLB and AD tau proteins contribute to neurodegeneration (Huang & Halliday 2013). On the molecular level LBD is further characterised by α-synuclein proteins in neurons, synaptic degeneration, as well as dopamine deprivation (Spillantini et al. 1998; Overk & Masliah 2014; H-J. Lee et al. 2014). In addition, symptoms such as hallucinations, behavioural abnormalities, rigid muscles and tremors, olfactory dysfunction, dysautonomia, depression, rapid eye movement, and disturbed sleep behaviour can be observed (Molano 2013).

(2) VD has been associated with focal, multi-focal or diffuse cortical and/or sub-cortical micro-infarcts, lacunas (in thalamus, frontobasal and/or limbic systems), hemispherical White Matter Hyperintensities (WMH), and in a few cases brain infarcts resulting from systemic, cardiac or small vessel disease affecting cognition, memory, and behaviour (Jellinger 2008). VD is usually difficult to distinguish from AD, for instance there is a similar distribution of WMH (Gootjes et al. 2004).

(3) FTD is an overarching term used for a range of clinically, neuropathologically and genetically heterogeneous disorders, and in total accounts for ca. 20 % of all dementia cases (Brun 1987; The Lund and Manchester Groups 1994; Goedert et al. 2012) (Pan & Chen 2013). FTD symptoms include: focal atrophy of the orbitomesial frontal and anterior temporal lobes, and can be accompanied by changes in social and emotional behaviour, already at ages 45-60 (Siri et al. 2001). In 1892, Arnold Pick firstly described a patient with these core symptoms (Rossor 2001).

(4) Other less common forms of dementia include Binswanger's disease (Caplan & Gomes 2010), Parkinson's disease (Goetz & Pal 2014), Huntington's disease (Roos 2010), Creutzfeldt-Jakob Disease (Creutzfeldt 1920; Panegyres et al. 2013), Multiple Systems Atrophy (Ahmed et al. 2012), Corticobasal degeneration (Grijalvo-Perez & Litvan 2014), and HIV-related cognitive impairment (Zhao et al. 2014).

A further distinction between cortical (mostly affecting the cerebral cortex) and subcortical (affecting areas beneath the cortex) dementia is usually made: AD, Creutzfeldt-Jakob, Parkinson's disease and Frontotemporal dementia are classified as forms of cortical dementia whereas cerebral palsy disorders (R. W. Lee et al. 2014), Huntington's, Wilson's (Li et al. 2014), White matter diseases (Weller et al. 2015), AIDS, and other rare encephalopathies are considered subcortical with dementia symptoms (Huber et al. 1986).

2.1 Clinical definitions

2.1.1 Clinical definition of AD

Probable AD is diagnosed when the patient fulfils the core clinical criteria for dementia and in addition, the following characteristics (McKhann et al., 2011):

1. Insidious onset: gradual onset of symptoms over a longer period of time (years)
2. Worsening of cognition
3. Amnesic symptoms including learning impairment and recall of learned information
4. Nonamnesic characteristics: language, visuospatial, executive dysfunction

Probable AD should not be diagnosed in cases of cerebrovascular disease, Lewy body dementia, frontotemporal dementia, aphasia, other neurological disease or medical comorbidity.

Memory loss is one of the first and most prominent symptoms of Alzheimer's disease. First memory problems start with subtle forgetfulness. Later, the capability of recall, recognition, registration and retention of information becomes more impaired; this includes difficulties with recognizing people and naming objects leading to disorientation and confusion: for instance, an individual with severe AD can get lost in his/her own home. As symptoms worsen all activities of daily living become affected and eventually full time care is required (Velayudhan 2011). Memory loss is hypothesised to be associated with the main pathological features of AD: accumulation

of amyloid-β plaques (Kam et al. 2013), and with the spread of neurofibrillary tangles from the entorhinal cortex to the hippocampus in the brain (Kerchner et al. 2012). Additionally, structural and functional brain changes (Jahn 2013), for instance atrophy in specific regions such as the posterior and medial temporal lobes (Smits et al. 2013), and in the hippocampus, affect memory severely (Kerchner et al. 2012).

Language becomes affected in AD with regard to oral, written expression, and selection of words, and also pronunciation in later stages (Abou-Saleh et al. 2010), and has been related to structural changes in the frontal lobe (Mega 2000).

Higher visual functions become gradually impaired: the primary visual cortex is initially less affected than the brain regions in which visual association tasks are performed (Prvulovic et al. 2002). The lower performance on visual tasks is associated with different areas in the brain: in early onset AD posterior atrophy has been observed and in late onset medial temporal lobe atrophy (Smits et al. 2013).

Executive functions are hampered by the disease: usually, AD patients encounter problems with executing planned movements, especially sequential execution of several steps, (re-)organising, and abstraction capabilities; all of these tasks depend on proper function of frontal lobe brain activity (Swanberg et al. 2004).

Disruptions of memory, language, spatial awareness, praxis and the associated brain activities can lead to severe mood swings and the behavioural symptoms associated with AD: including delusions, hallucinations, depression, aggression, dis-inhibition, agitation, and sleep disturbances. Sleep patterns have been found to be more fragmented, causing people to wake up more often during the night, and also rapid eye movement sleep is

disturbed (Petit et al. 2005). Some changes in eating behaviour have been observed; however, they do not occur as frequently as in other dementias (Ikeda 2002). Hallucinations or delusional thinking occur later in the disease course and have been associated with cognitive decline (Wilson 2000). Cognitive and behavioural problems dominate the patient's life and he/she requires eventually full time care. On MRI scans whole brain atrophy, hippocampal atrophy are prominent features and on PET scans increased amyloid burden can be observed. In the late stages, $A\beta^{1-42}$ has accumulated in the brain alongside with tau-pathology, leading to dementia.

2.1.2 Clinical definition of MCI

Exact boundaries between normal cognition and MCI and between MCI and dementia are difficult, and clinical judgement is required for such distinctions (Albert et al., 2011).

1. There is evidence of a change in cognition
2. Increasing impairment in one or more cognitive domains including memory, executive function, attention, language, and visuospatial skills, and episodic memory (the ability to learn and retain new information).
3. Persons with MCI are usually still performing in day-to-day tasks but less efficiently.
4. MCI requires evidence of intra-individual change.

There are known limitations in the search for accurate biomarkers. The diagnosis of AD and MCI can take place on a biomarker spectrum and in some cases can be inaccurate even when using standardised tests. The challenge is avoiding misclassification, thus a

combination of biomarkers such as imaging, clinical cognitive test, gene expression, and protein data could be more accurate for diagnosis. In the current study, individuals with MCI reported problems with memory but performed normal in activities of daily living as specified in the Petersen's criteria for amnestic MCI (Peterson et al. 1999; Peterson et al 2001).

2.1.3 Definition of Preclinical AD

Preclinical AD describes a newly defined stage of the disease reflecting measurable biomarker changes that could occur a number of years before symptoms affecting memory, thinking or behaviour. However, such changes are not used as diagnostic criteria yet. They are regarded as complementary indicators (biomarkers) under investigation to better define AD in future and move the efforts toward earlier intervention, and finally, toward the prevention of AD dementia (Sperling et al., 2011). The preclinical phase starts usually more than a decade before first symptoms occur (Jack et al. 2013), sometimes already 15 years before. During this period several structural, functional, and biochemical events are occurring along with neuro-cellular instabilities. Markers of these changes were detected in plasma and cerebrospinal fluid (Gold et al. 2012; Lunnon et al. 2013), fibrillar amyloid deposition (Morris et al. 2010), cognitive event-related potentials (Olichney & Hillert 2004), mitochondrial dysfunction (Maruszak & Żekanowski 2011), and immune activation (Lunnon et al. 2012).

2.1.4 Definition of Prodromal AD

Prodromal AD is referred to as the pre-dementia stage of AD in which clinical amnestic symptoms and biomarker evidence of AD progression are detectable (Chertkow et al., 2013) (Figure 1). At the minimum one abnormal AD progression biomarker has to be found for reclassification of a patient from MCI (a risk state) to prodromal AD (a stage of AD).

In the prodomal phase, subtle cognitive and behavioural problems can occur, often in executive functions, motivation, or affective behaviour. The patient remains independent but often shows a set of symptoms referred to as MCI. About 3 to 19 percent of those aged 65 and older meet MCI criteria (Gauthier et al. 2006), and 50%-70% of them develop dementia (Drago et al. 2011). Nevertheless, not all patients with MCI develop AD, some stabilize or even improve their mental abilities.

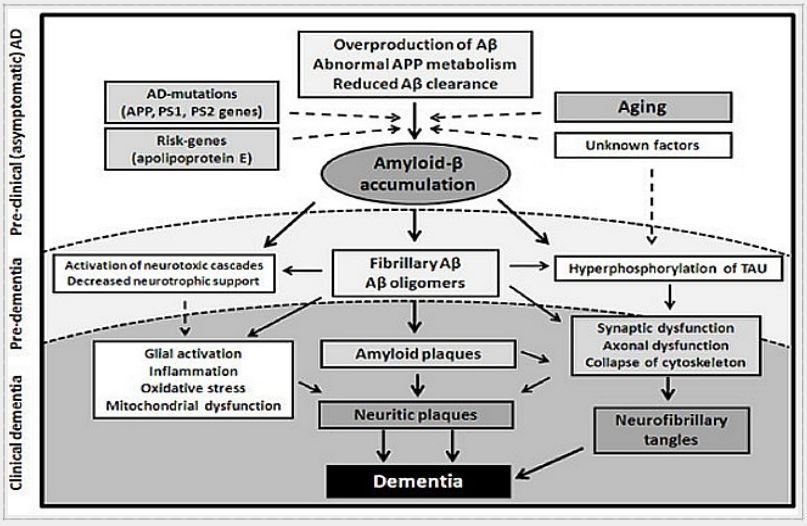

Figure 1: Stages of AD related to molecular events The course the disease is characterised by three phases: (1) preclinical, (2) prodomal, (3) symptomatic, phase with dementia present(Adapted from Forlenza et al., 2010)

2.2 Neuropsychiatric scales

Neuropsychological tests remain the most prevalent tool for AD diagnosis (Jahn 2013). Since 1984 cognitive standardized assessments and global measurements of dementia are used: the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog) (Pea-Casanova 1997), the Clinical Dementia Rating (CDR) (Hughes et al. 1982), and the Mini-Mental State Exam (MMSE) (Folstein et al. 1975), and many other others. These tests measure many aspects of memory, language and spatial orientation skills.

2.2.1 ADAS-cog

The ADAS tests have been developed to measure the severity of the most important symptoms of Alzheimer's disease. A subscale of the ADAS-cog is frequently employed for cognitive assessments in clinical trials comprising 11 tasks covering: memory, language, praxis, attention. Its scoring system ranges from 0-70 points where higher scores means worse cognitive performance.

2.2.2 Clinical Dementia Rating

The CDR-Global is a combined functional scale ranging from 0-18 points, and the CDR-Sum Of Boxes (CDR-SOB) comprises six different assessment categories ranging from 0-3 points. Higher scores are associated with greater cognitive impairment. The ADAS-cog and the CDR-SOB provide more detailed information than the global CDR score

(Lynch et al. 2006). CDR is important to differentiate MCI from AD, it has been found suitable for identifying subgroups of individuals with MCI who have more widespread neurodegeneration and are more likely to progress to probable AD (Chang et al., 2011).

2.2.3 Mini-Mental State Exam

The MMSE is the most commonly used assessment in early AD stages, and is also often used for measuring AD progression and severity. The questions are focused on memory and orientation ranging from 0-30 points. Lower scores are associated with greater cognitive impairment. Patients with AD often lose up to four points yearly (on average) measured by MMSE. Doctors usually consider the MMSE performance when administering drugs. The MMSE boundary for AD is described with a score of 20 to 24 suggesting mild dementia, 13 to 20 moderate dementia, and less than 12 indicates severe dementia. On average, the MMSE score of a person with Alzheimer's declines about two to four points each year. A score of 24 yielded a sensitivity of 0.58 and a specificity of 0.98 in detecting probable and possible Alzheimer's disease across ethnicities (Spering et al., 2012). The MMSE boundary for MCI is 25-30 = Normal / 19-24 = Borderline / <19 = Impaired (Folstein MF, Folstein SE, and McHugh PR, 1975)

General considerations regarding cognitive testing

A bad performance in a cognitive test does not necessarily indicate persistent dementia, it remains the possible that the patient suffers from other conditions which impact his/her cognitive performance temporarily. The daily mental fitness could vary

depending on mood, pain, or health problems.

Additionally, a disadvantage of cognitive tests is that education, cultural background, and (speech) disabilities can severely influence the test results. A person with lower education might be misclassified of having dementia.

4 Pathology

4.1 Molecular pathology

There are two major pathological features that characterize AD: (i) the presence of large extracellular 'neuritic plaques' composed of aggregated amyloid β peptide 42 (Alzheimer 1907; Glenner & Wong 1984; Findeis 2007), surrounded by abnormally formed neuronal processes or neurites (Perl 2010), and (ii) the accumulation of intracellular 'neurofibrillary tangles' which are cytosolic inclusions mainly consisting of hyperphosphorylated and presumably abnormally cleaved tau protein, a microtubule-associated protein (Grundke-Iqbal et al. 1986; Huang et al. 2001) (Figure 2).

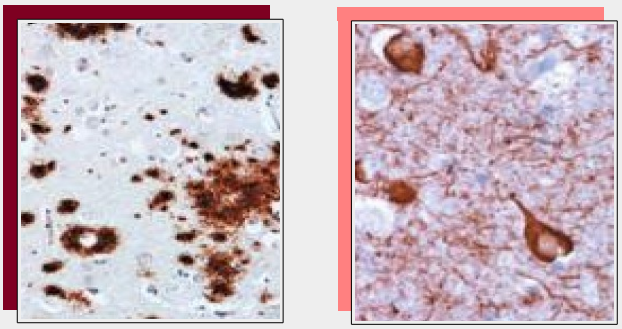

Figure 2: Senile plaque (left) and neurofibrillary tangle (right). (Adapted from (Ingolia 2014)

Aβ is produced in the brain from the Amyloid Precursor Protein (APP). APP is a single-pass transmembrane protein with a large extracellular domain containing two heparin-binding sites (Mok et al. 1997) (O'Brien & Wong 2011); the gene which expresses the APP protein is located on chromosome 21. Multiple alternative splicing isoforms of APP have been identified in humans with from 365 to 770 amino acids length. Certain isoforms are preferentially expressed in neurons and changes in their ratio have been related to Alzheimer's disease (Matsui et al. 2007). The exact physiological role of APP is still not known but it has been shown that APP is capable of modulating cell growth and survival, motility, neurite outgrowth (Pung et al. 2013), intracellular trafficking, and proteolytic processing (Thinakaran & Koo 2008). APP is the precursor to the amyloid β protein, a 38-43-amino acid residue peptide which is the core of the "*amyloid cascade hypothesis*".

4.1.1 The Amyloid Cascade Hypothesis

Although the current prevailing explanation for AD (the so-called 'amyloid cascade hypothesis') is based on the strong correlation between the development of the disease and the appearance of Aβ deposition in the brain of patients, the views on the exact role of Aβ still remain obscure and somehow controversial. The amyloid cascade hypothesis initially proposed by Hardy & Higgins (1992) postulates two different pathways of APP processing in the cell. Interestingly, only one of these pathways is associated with the

generation of toxic amyloidogenic Aβ deposits (Figure 3).

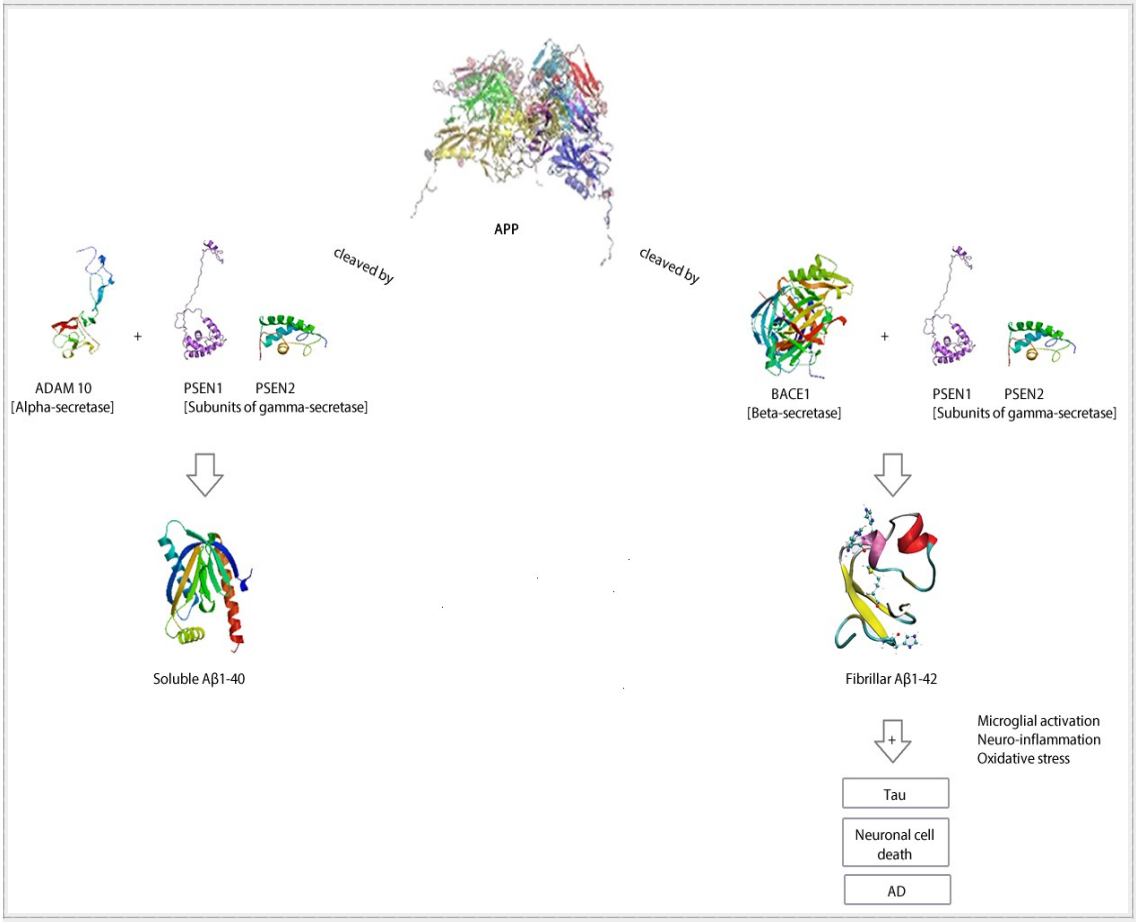

Figure 3: The amyloid cascade hypothesis: the mechanism of A- β 42 mediated neurotoxicity; the pictures correspond to crystal structures of protein subunits deposited in 'The Worldwide Protein Data Bank' [http://www.wwpdb.org/]

APP can be involved in a non-amyloidogenic (i.e., non-pathological) pathway that involves the sequential action of α- and γ-secretases on the cell surface. This pathway generates a harmless version of Aβ. However, in the amyloidogenic (i.e., pathological) pathway, APP is proteolytically processed and reinternalized through clathrin-coated pits into endosomal (or lysosomal) compartments that contain the proteases BACE1 (β-secretase) and γ-secretase (Vassar et al. 1999; O'Brien & Wong 2011; Nakayama et al.

2013). First, BACE1 (the neuronal β-secretase), a transmembrane aspartic protease, cuts APP at the positions +1 and +11 sites of Aβ (Cai et al. 2001). Second, the remaining APP C-terminal fragment is cut by γ-secretase complex at one of several varying sites from positions +40 to +44, generating the APP intracellular domain and Aβ peptides (such as Aβ1-40 or Aβ1-42) which eventually will reach the extracellular milieu. It remains unknown what signals decide which proteolytic processing (α+γ *versus* β+γ) APP will undergo in the cell. Of note, the Aβ1-42 peptide has been found to be the most abundant proteolytic fragment and also the most toxic. Aβ1-42 accumulation (or the excessive accumulation of Aβ1-42 *versus* Aβ1-40) is therefore the most deleterious event in the current molecular hypothesis underlying the etiology of AD.

In the amyloidogenic pathway γ-secretase, is a multiprotein complex composed of presenilin 1 (PSEN1), presenilin 2 (PSEN2), nicastrin, APH-1, and PEN-2. This complex mediates the cleavage of transmembrane protein such as APP and NOTCH (De Strooper et al. 1999). The exact role of the different subunits of the γ-secretase complex is still not fully understood but it is hypothesized that changes in NOTCH-proteolysis mediated by γ-secretase may be involved in the pathogenesis of AD (Woo et al. 2009).

An increase in BACE1 activity has been reported in brain tissue samples of patients with sporadic AD (Hampel & Shen 2009). Up-regulated BACE1 activity could be a consequence of the activation of the transcription factor HIF-1α or a general augmentation of oxidative stress in the brain (Chami & Checler 2012). It is still unclear whether monomeric, oligomeric, or fibril forms of toxic Aβ peptides are the most toxic

to neurons. Also, it has been shown that Aβ can be toxic extracellularly and not only when accumulates in the lumen, it binds strongly to tau in solution which could be a precursor event to later self-aggregation of both molecules (Guo et al. 2006).

Neurofibrillary tangles, formed by aberrantly paired helical filamentous aggregates of the microtubule-associated protein tau, are hypothesized to cause neural cell death and lead to the symptoms associated with AD (Chesser et al. 2013). They stabilize microtubules during axonal transport and also enhance neuronal processes outgrowth (Kowall & Kosik 1987). Hyperphosphorylated Tau aggregates in filaments and loses its microtubule-binding and -stabilizing functions leading to neuronal death. Tau hyperphosphorylation has been reported to be mediated by several protein kinases including GSK3 (F. Hernández et al. 2010), CDK5 (Hernández & Avila 2008), p38 (Zhu et al. 2000), JNK (Ploia et al. 2011), PKA (Tanaka et al. 2000), PKC (Tanaka et al. 2000), CAMKII (Wang et al. 2007), CKII (G. Yu et al. 2013), and MARK (Gu et al. 2013). In total, up to 79 serines/threonines have been reported to be phosphorylated in the longest tau mammalian isoform of 441 amino acids (Figure 4).

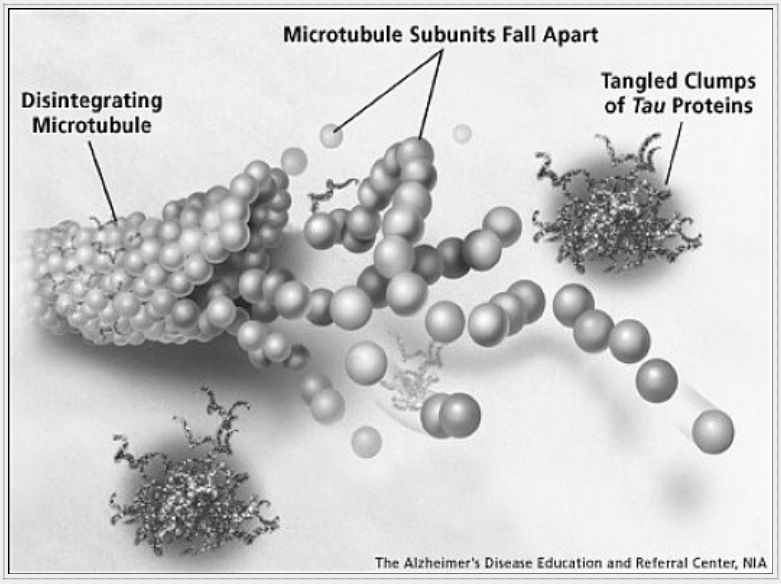

Figure 4: Disintegrating microtubule and tau protein tangles (Adapted from ADEAR: "Alzheimer's Disease Education and Referral Center of the National Institute of Ageing.")

4.1.2 Pathways implicated in the initiation and progression of AD

Recent novel insights in key-pathways lead to a deeper understanding of AD: (1) the innate immune system, (2) cholesterol metabolism, (3) endosomal vesicle recycling, (4) autophagy, and most recently (5) protein ubiquitination provide a more holistic view of AD pathogenesis involving a number of different cell-types and mechanisms.

(1) The innate immune system

In the central nervous system microglia, a type of glial cell with macrophagic function, are capable of responding to injury and infection. On their surface Toll-like receptors (TLR) are expressed which are critical for eliciting an innate immune response and triggering the adaptive immune system, recognising pathogen-associated molecular patterns, and also Aβ (Boutajangout & Wisniewski 2013). TLR respond to fibrillar Aβ

by accumulating toxins and increasing cytokine production leading to neuro-inflammation, and -degeneration. Other stimuli that lead to elevated microglia activity include: oxidative stress, proinflammatory cytokines (a vicious cycle), glutamate receptor antagonists, cell necrosis factors, lipopolysaccharide, and changes in extracellular potassium. It is hypothesized that also environmental toxins and endogenous proteins induce microglial overactivity (detectable with imaging methods) leading to neuroinflammtion. Drugs, acting upon inflammatory processes and thus microglia could potentially influence the AD progression (Block et al. 2007; Schott & Revesz 2013). There could be other feedback loops, yet to be identified, between Aβ and microglia involved in the process of inflammation in AD (Meyer-Luehmann et al. 2008). One practical implication of the effect of inflammation has been shown in late-onset AD where anti-inflammatory drugs, taken long before disease onset, have been shown to be beneficial in decreasing the disease risk (Etminan et al. 2003). Another innate immune receptor on microglia is TREM2. Defects in this gene lead to chronic neuroinflammation and microglial activation by increase of cytokine production and secretion (Boutajangout & Wisniewski 2013). Other genes that are associated with microglia and increased risk of late-onset AD are CR1, CD33, and MS4A4A/MS4A6A (Hollingworth et al. 2011; Gandy et al. 2013; Proitsi et al. 2014). These studies suggest that modification of microglial function in AD is a potential therapeutic target.

(2) Cholesterol metabolism

High levels of cholesterol in the blood (hypercholesterolemia) and abnormal cholesterol processing are strongly related to toxic amyloid plaque formation and tau hyperphosphorylation; possibly cholesterol oxidation products "oxysterols" are contributing to toxic deposits (Gamba et al. 2012).

(3) Endosomal vesicle recycling

Neurotransmitters are transported in vesicles (Figure 5); the supply of vesicles comes from the cell-body. Remarkably, the neurons can sustain high rates of synaptic transmission without using up their supply of synaptic vesicles relying on endocytic recycling of synaptic vesicle membranes (Saheki & De Camilli 2012). However, this local recycling at nerve terminals requires them to be structurally and chemically intact which is probably not the case in AD leading to failure of synaptic transmission (Overk & Masliah 2014). There is evidence that defective vesicle recycling or exo-endocytic coupling can be related to deficits in short-term synaptic plasticity, memory, and cognitive performance (Murthy & De Camilli 2003; Lambert et al. 2013).

Additionally, other important AD molecules are trafficked in vesicles, as previously mentioned: "APP is reinternalized through clathrin-coated pits into endosomal (or lysosomal) compartments that contain the proteases BACE1 (β-secretase) and γ-secretase. This process results in the production of Aβ (Aβ1-40 or Aβ1-42) which eventually will reach the extracellular milieu. Endocytic mechanisms can be also found in other cell types but in neurons there are some specific adaptations (Saheki & De Camilli 2012), especially in AD implicated cells.

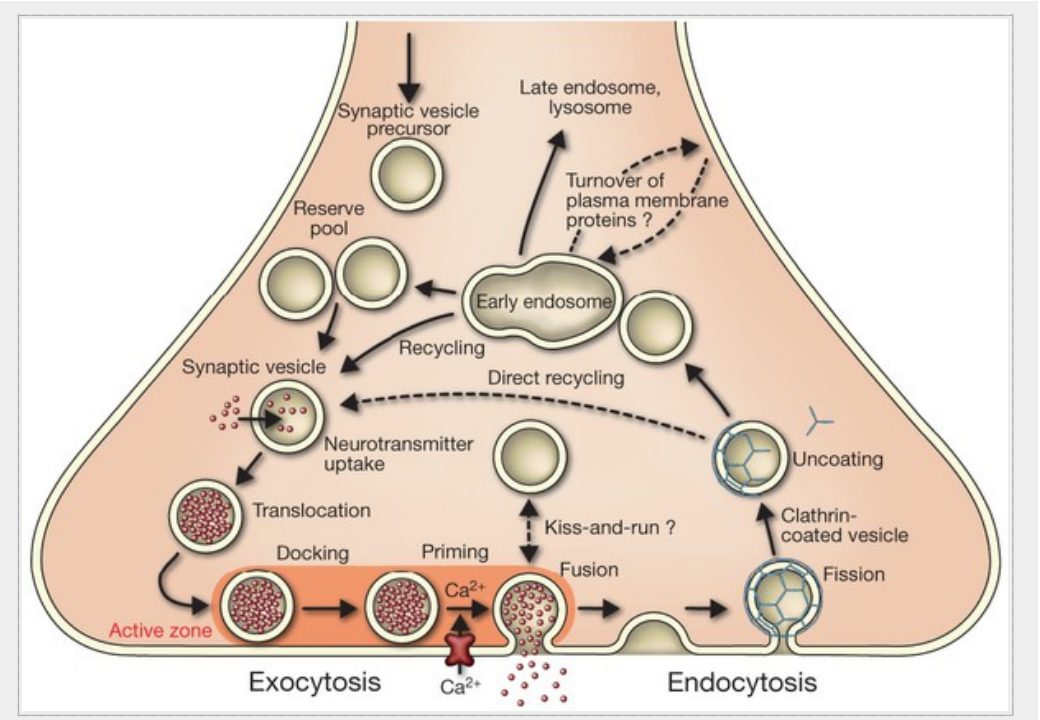

Figure 5: Endosomal vesicle recycling (Adapted from Jahn & Fasshauer 2012)

(4) Autophagy

Autophagy is usually a protective, highly conserved eukaryotic cellular recycling process (Parzych & Klionsky 2014), disruption of which can lead to various neurodegenerative diseases, inclusive AD. Autophagy functions by degrading toxic and damaged organelles, macromolecules such as protein aggregates, and eventually this cytoplasmic cell waste is destroyed in lysosomes; defective lysosomal acidification contributes to proteolytic failure and loss of other important cell functions (Wolfe et al. 2013). Recent research suggests that defects in the autophagosomal fusion with lysosomes lead to insufficient protective autophagy in AD (Barnett & Brewer 2011).

(5) Protein ubiquitylation

Ubiquitin and Ub-like modifiers are small proteins that upon covalent attachment regulate protein functions in eukaryotic organisms; they can be determined in most cellular pathways and are a key-players in disease onset. Ub requires three types of enzymes: ubiquitin-activating enzymes, ubiquitin-conjugating enzymes and ubiquitin ligases. Ubiquitylation might play an important role in AD by regulating the development of the immune system and immune responses: initiation, propagation and termination (Bhoj & Chen 2009). A number of ubiquitin ligases prevent auto-immune attacks. Their dysfunction is implicated in disease but the exact role in AD remains to be elucidated.

4.2 Structural and functional brain changes

The most remarkable visible feature, on MRI scans or post-mortem, is whole brain atrophy, including many important structures: hippocampus, cerebral-, and entorhinal cortex (Figure 6). Atrophic changes in the brain are associated with worse cognitive performance (Rayaprolu et al. 2013), and cognitive decline (L. Smits et al. 2013). At early stages of AD, atrophy and metabolic changes emerge especially in posterior cortical regions and also in medial temporal regions (Velayudhan et al. 2013).

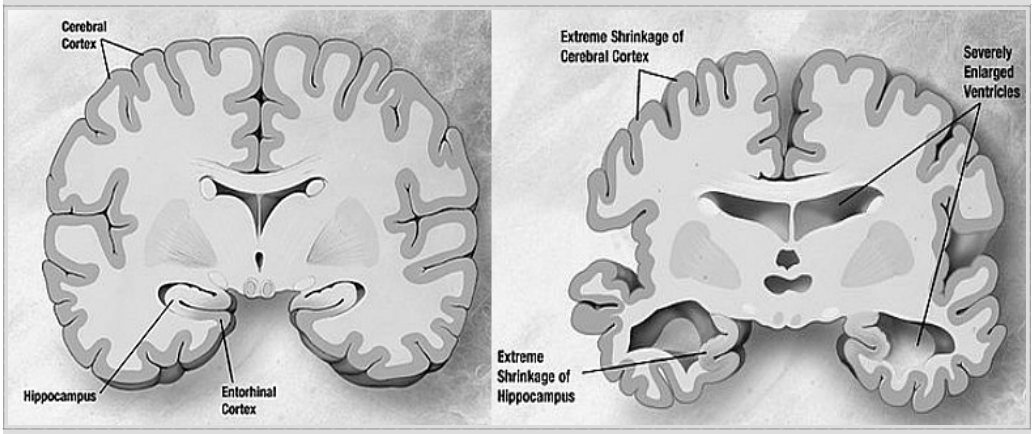

Figure 6: Healthy brain versus AD brain (Adapted from the National Institute of Ageing, 2002)

Other pathological structure-related features include granulovacuolar degeneration and eosinophilic rodlike bodies (Hirano bodies), and the loss of synaptic connectivity which clearly has a huge impact on cognitive function (Buckner et al. 2005).

5 Genetics

Around 95 % of the AD cases occur sporadic (late-onset AD). However, among the 30% proportion of unknown cases, new common variants have been recently detected; most of these genes are associated with very small relative risk of AD (Figure 7 & 8).

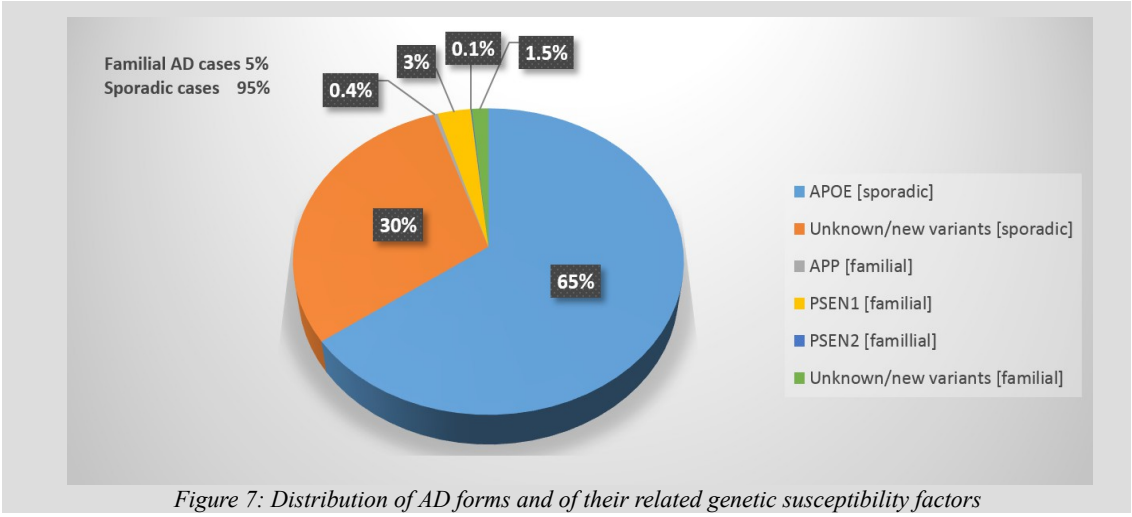

Figure 7: Distribution of AD forms and of their related genetic susceptibility factors

Mutations in the *APP*, *PSEN1*, *PSEN2*, *TREM2* (Triggering Receptor Expressed on Myeloid cells 2) and *CSF1R* (Stimulating Factor 1 Receptor) genes occur in less then 5% of AD cases (Figure 8).

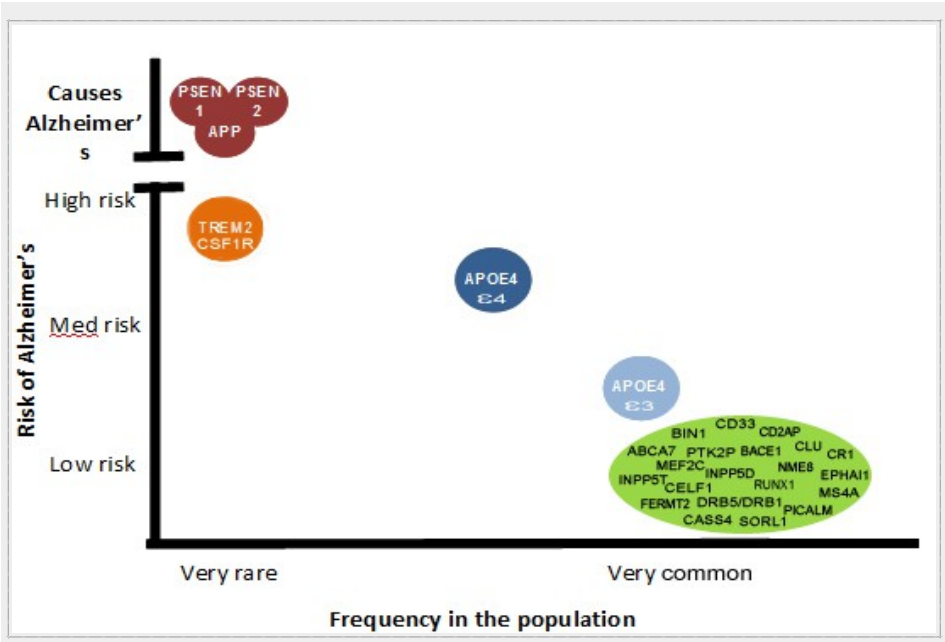

Figure 8: Rare and common genetic loci with their associated risk in AD (Adapted from Manolio et al., 2009, and updated with recently discovered variants)

The onset in these rare genetic cases is much earlier, affecting people already in their 40s in contrast to the cases with increased risk due to common susceptibility genes with onset above 65. Rare mutations have usually a large disease effect (Table 1). Individuals with rare mutations deteriorate very fast, whereas late-onset cases develop the disease over years, probably even decades.

	Genetic disease	**Genetic susceptibility**
Onset	early	late
Genetic component	mutation disease related	mutation risk related
Mutation frequency	rare	common
Inheritance	mendelian	complex
Effect on individual	large	weak
Effect on population	weak	large
Epigenetic influence	small	large

Table 1: Genetic disease versus susceptibility (Manolio et al. 2009)

5.1 Early Onset Familial AD

Rare genetic variants which are associated with early onset AD include: *APP, PSEN1, PSEN2, TREM2*, and *CSFR1*. Their functions are associated with APP- processing (*APP, PSEN1, PSEN2*) and immune responses (*TREM2, CSFR1*). Mutations in *APP, PSEN1* and *PSEN2* have been mainly associated with early onset AD (<60 years) in familial AD cases (Richard & Amouyel 2001). However, new evidence has been found which relates also late onset familial AD to these rare variants (Goate et al. 1991; Cruts et al. 1998).

Early onset familial cases are due to a strong genetic element: autosomal dominant missense mutations (a single nucleotide change results in a codon that codes for a

different amino acid), in individuals younger than 60 years, in the Amyloid Precursor Protein (APP) and Presenilins 1 and 2 (PSEN 1 and 2) genes (O'Brien & Wong 2011) (De Strooper et al. 1999) (Cruchaga et al. 2012), and a rare autosomal recessive missense mutation in the *TREM2* gene.

Clinically and neuropathologically there are some differences between early onset and late onset AD, such as increased levels of C-reactive protein, creatine, and blood urea nitrogen. C-reactive protein is produced by the liver as a response to injury, infection, and inflammation which could have an impact on cognitive decline in AD (Panegyres & Chen 2013).

However, some neuropsychological findings suggest that there are similarities between early onset and sporadic AD such as amnesic and dys-executive syndrome which can be observed in both (Greene et al., 1995).

5.1.1 APP- mutations

There are at least 36 APP gene missense mutations usually clustering around the γ-secretase cleavage site, but also around the BACE1 cleavage site in primary cultures of human neurons (Guerreiro et al. 2013), resulting in early-onset, autosomal dominant AD; these mutations account for 10% to 15% of early-onset familial AD (De Jonghe et al. 2001). Of note, it is hypothesized that APP plays also a role in sporadic AD and trisomy 21 (Down's syndrome). People with Down's syndrome produce increased levels of APP (and therefore Aβ 1-42) as a consequence of a gene duplication on chromosome 21, leading to early AD. These patients can develop dementia and AD pathology already

in their 20s (Abou-Saleh et al. 2010; Cruts et al. 2012). Interestingly, there are also more common AD variants within genes such as APOE, SORL1, RUNX1, BACE1, ALDH18A1, which are also associated with Down's syndrome (Sparks et al. 2013).

5.1.2 PSEN-mutations

In the past rare PSEN mutations have been associated with early onset familial AD: these mutations have been found in two genes, PSEN1 on chromosome 14 and PSEN2 on chromosome 1. The incidence of PSEN 1 is very low with 3%, and for PSEN2 even lower with 0.1% of all reported AD cases worldwide (Patel et al. 2011). According to the "Alzheimer Disease & Frontotemporal Dementia Mutation Database" (Richard & Amouyel 2001), at least 185 mutations in PSEN1 have been mapped while 14 mutations have been found for PSEN2. Mutations in the presinilin genes facilitate proteolysis of APP by γ-secretase to form toxic Aβ in transgenic mice with APP mutations who develop plaques as they age; genetically-modified mice (Tg2576 crossed with PS1M146L) with both the human APP and PSEN genes show plaques at a much younger age (Holcomb at al. 1998). These mice had enhanced amyloid deposition, behavioural symptoms, less performance in the water maze, and reflect largely disease pathogenesis. Such remarkable findings have led to the hypothesis that the loss of presenilin function is the major causative factor for AD pathogenesis (Hardy & Higgins 1992).

5.1.3 TREM2-mutations

Recently, rare mutations in the *TREM2* gene (affecting around 1-3% of AD patients) have been found to be associated with AD (Jonsson et al. 2013; Guerreiro et al. 2013; Ruiz et al. 2013). *TREM2* is found in the membrane of cell types linked with immune function, including macrophages, dendritic cells, osteoclasts, and microglia. TREM2 is expressed at high levels in white matter but also in the hippocampus, medulla, putamen, neocortex and at lower levels in the cerebellum (Forabosco et al. 2013).

The rs75932628 variant

The variant rs75932628, predicted to cause a R47H substitution in *TREM2*, increases the AD risk with an odds ratio similar to that of the APOE ε4 allele, ranging from 2.9-5.1 (Guerreiro et al. 2013; Jiang et al. 2013; Forabosco et al. 2013; Jonsson et al. 2013; Gonzalez Murcia et al. 2013). AD onset occurs ~3.5 years earlier in carriers of the rs75932628 variant than in non-carriers and the carriers exhibit mature and diffuse amyloid plaques already in an early disease stage (Jonsson et al. 2013). Interestingly, the variant is associated with lower cognitive functioning in AD but also in non-demented subjects, above age 80 (Guerreiro et al. 2013; Jonsson et al. 2013; Benitez et al. 2013).

There was also evidence for an association of R47H with FDT and Parkinson's disease (Fenoglio et al. 2007; Guerreiro et al. 2013; Jiang et al. 2013; Ruiz et al. 2013). Another study confirms the strong association of R47H with AD but did not find an association with FTD (Forabosco et al. 2013).

In the Icelandic population R47H has been associated with a lack of containment of neuroinflammation. Further verification is needed in other racial and ethnic groups to

verify if the harmful mutation has the same effect (Guerreiro et al. 2013).

Other AD-specific variants in the TREM2 gene

In addition, six *TREM2* variants were observed in AD subjects but not in controls: H157Y, R98W, D87N, T66M, Y38C, and Q33X. These could contribute to AD pathogenesis (Rayaprolu et al. 2013). More large-scale genetic screenings to discover other functional variants in *TREM2* remain to be performed.

TREM2 and Nasu-Hakola disease

Of interest, mutations in *TREM2* appear to play a role in Nasu-Hakola disease by triggering the production of constitutive inflammatory cytokines (Cella et al. 2003; Paloneva et al. 2003; Bianchin et al. 2010; Bock et al. 2013). Nasu-Hakola, a progressive presenile inflammatory neurodegeneration, disease is a rare mendelian disease characterized by bone reabsorption dysfunction and chronic inflammatory neurodegeneration, leading to death at ages in the 40s or 50s. The exact pathomechanism underlying Nasu-Hakola disease remains unclear but microglial clearance failure appears to be implicated (which seems to be also related to late-onset AD and to malfunction of TREM2 gene expression).

TREM2 and TYROB

Early studies have found that *TREM2* together with *TYROBP* (former *DAP12*) expression deficiency is associated with impaired osteoclast differentiation and osteoporosis (Paloneva et al. 2003). The *TREM2* and *TYROB* receptor complex plays also an important role in immune cells such as microglial cells which protect from foreign pathogens and remove dead cells and other debris in the brain (Ito & Hamerman

2012; Jiang et al. 2013). However, the exact function of the *TREM2* - *TYROB* complex remains to be elucidated.

TREM2 and phagocytosis

TREM2 is associated with Alzheimer's disease, multiple sclerosis, and motor neuron disease, supporting the hypothesis that these diseases have common underlying pathways implicating microglia (Forabosco et al. 2013). Experimental evidence has shown that an increase of TREM2 expression enhances microglial clearance of amyloid plaques in mice via a process known as phagocytosis (Melchior et al. 2010; Forabosco et al. 2013; Rayaprolu et al. 2013; Sierra et al. 2013). These results have led to the hypothesis that TREM2 has a protective role in AD by suppressing inflammatory cell responses as well as promoting phagocytosis of Aβ in damaged neurons. TREM2 could be potentially used in vaccine-based autoimmune (anti-amyloid) treatments by decreasing the severity of auto-immune responses (Melchior et al. 2010).

5.1.4 CSFR1-mutations

CSFR1 a human cell-surface protein of the *CSF1R* gene, is a receptor for a cytokine called colony stimulating factor 1 (CSF1) and IL34 (Guerreiro et al. 2013), located on chromosome 5. CSF1 controls the production, differentiation, and functioning of macrophages.

CSF1R missense mutations are rare and associated with Hereditary Diffuse Leukoencephalopathy with Spheroids (HDLS) and sometimes diagnosed together with AD. HDLS is characterized by huge axonal swellings (spheroids) within the CNS white matter, executive dysfunction, memory decline, personality changes, motor impairment,

and seizures (Wang et al. 2012; Rademakers, et al. 2012); the age of onset is below 60 years.

Elevated levels of CSF1R1 are found in hyperactive microglia in Alzheimer's disease and after brain injuries (Sundal et al. 2012). CSF1R and CSF1 may be involved in breast cancer (Mitrasinovic et al. 2005).

CSF1is a key regulator of the monocyte/macrophage lineage. The administration of human recombinant CSF1 improves memory in a transgenic mouse model of Alzheimer's disease (Tamimi et al. 2008).

Most cases of Alzheimer's disease are not hereditary but sporadic. It is still unclear how sporadic cases of AD arise. To date, research efforts have somehow focused on hereditary cases even if they represent a small subset of the total cases of AD. Rare variants contributing to early-onset AD have a larger effect size on the individual in comparison to common variants which usually have smaller effect sizes. Loci of common variants can be identified by means of DNA sequencing methodology and computationally processed in a GWAS (Genome-Wide Association Study). More work has to be done on sporadic early onset which includes screening of larger patient pools for rare mutations.

Consistent with the amyloid cascade hypothesis, it can be concluded that mendelian genes are involved in Aβ production, which is controlled and can even be reduced by certain protective variants. Some of these genes play a role in major biological responses such as neuronal survival, plasticity, innate and adaptive immune responses, and glucose

metabolism (Saura et al. 2004; Lanier 2009; Fowler et al. 2011; Mosconi 2013). It is thought that increasing understanding of their roles in both normal and pathological conditions would eventually lead to the development of drugs able to manage, control, or cure Alzheimer's disease.

5.2 Late Onset Sporadic AD

In the past most of the studies focused on *APOE,* however, recently many more common risk variants have been identified. In this section, the *APOE* isoforms and their functions are described in detail and afterwards an overview of the recently discovered variants is provided.

5.2.1 APOE-variants

There are three variants of the human *APOE* gene, which is located on chromosome 19: the polymorphic alleles (isoforms) *ε2*, *ε*3, and *ε*4 with a population frequency of 8.4%, 77.9%, and 13.7%, respectively. At least one *APOEε*4 allele is found in ca. 37% of AD patients; a very common genetic variant (*APOE* genotype ε3, ε4) can be found in ca. 65% of the cases (Luo et al. 2013). There are there isoforms representing variants at amino acids 112 and 158 (Table 2).

Isoform	Isoform-specific amino acid difference		Allele frequency (%)		Genotype frequency (%)		Risk (%)
	112	158	General	AD	Genotype	%	
APOEε2	Cys	Cys	8.4	3.9	*ε2/ε2* *ε2/ε3*	1 20	0.08 Protective 3.2 variant
APOEε3	Cys	Arg	77.9	59.4	*ε3/ε3* *ε3/ε4*	60-65 21	5.1 Intermediate 18 risk
APOEε4	Arg	Arg	13.7	36.7	*ε4/ε4*	2	65 High risk

Table 2: APOE isoforms, differences, allele & genotype frequencies, and functions (Richard & Amouyel 2001)

Carriers of the *APOE ε*4 allele have lower Cerebro-Spinal Fluid (CSF) A*β*1-42 levels, elevated tau levels, and more brain or hippocampal atrophy than non-carriers (Raber et al. 2004; Hostage et al. 2013). These factors increase the risk for developing late onset AD (Vemuri et al. 2010).

*APOEε*4, synthesized by astrocytes, supports transport of cholesterol into cells, immune response, and controls repair after brain injury. *APOEε*4 binds to cell-surface receptors delivering lipids, and to A*β*, leading to synaptic malfunction and neurodegenerative cascades underlying AD (Liu et al. 2013); this allele is further associated with increased risk of cerebral amyloid angiopathy and age-related cognitive decline in normal ageing.

5.2.2 Recent common risk variants

Recently, 21 common risk variants have been discovered (Table 3).

1. ABCA7 (Hollingworth et al. 2011) (Reitz et. Al 2013)

2. BIN1 (Miyashita et al. 2013) (Thambisetty et al. 2013)

3. CASS4 (Mayeux et al. 2013)

4. CELF1 (Hinney et al. 2014)

5. CD2AP (Karch & Goate 2014) (Hollingworth et al. 2011)

6. CD33 (Naj et al. 2011) (Hollingworth et al. 2011) (Lambert et al. 2013)

7. CR1 (Aso & Ferrer 2013) (Lambert et al. 2013)

8. CLU (Lambert et al. 2009) (Harold et al. 2009) (Lambert et al. 2009) (Lambert et al. 2013) (Thambisetty et al. 2013) (Chapuis et al. 2013)

9. DSG2 (Lambert et al. 2013) (Karch & Goate 2014)

10. EPHA1 (Naj et al. 2011) (Hollingworth et al. 2011) (Lambert et al. 2013)

11. FERMT2 (Karch & Goate 2014)

12. HLA-DRB5/DRB1 (Lambert et al. 2013) (Naj et al. 2011) (Killick et al. 2014)

13. INPP5D (Lambert et al. 2013)

14. MEF2C (Karch & Goate 2014)

15. MS4A6A (Harold et al. 2009) (Hollingworth et al. 2011) (Naj et al. 2011) (Lambert et al. 2013) (Gandy et al. 2013) (Gandy et al. 2013)

16. NME8 (Karch & Goate 2014)

17. PICALM (Harold et al. 2009)

18. PTK2B (Karch & Goate 2014)

19. SLC24H4-RIN3 (Karch & Goate 2014)

20. SORL1 (Reitz et al. 2013) (Lambert et al. 2013) (Aso & Ferrer 2013)

21. ZCWPW (Karch & Goate 2014)

Table 3: Recent common risk variants

The discovery of these new risk variants has implicated several biological pathways, including the immune system, processing of cholesterol and lipids in the brain, and endocytosis, a process which removes toxic amyloid-β protein from the brain. According to their involvement in cellular functions and pathways, the different common variants genetic risk factors can be classified (Table 4).

1. Lipid/ cholesterol processing (Carter 2011):
APOEε4 (Malpass 2013), ABCA7 (Crehan et al. 2012), CLU (Poirier 2000), SORL1

2. Immune response/microglial activation/inflammation:
APOEε4, ABCA7, CD33 (Jones et al. 2010), CR1 (Harold et al. 2009) (Rosenthal & Kamboh 2014), INPP5D (Lambert et al. 2013), MEF2C (Lambert et al. 2013), HLA-DRB5/DRB1 (Lambert et al. 2013)

3. Endocytosis/endosomal vesicle recycling:
APOEε4 (Meurs et al. 2012), BIN1 (Rhinn et al. 2013) (Reitz et al. 2013), CD2AP (Carter 2011), CLU

(Carter 2011), EPHAI1 (Hollingworth 2011), MS4A6A, PICALM (Carter 2011), SORL1 (Karch & Goate 2014)

4. Amyloid processing:
SORL1 (Lambert et al. 2013), CASS4 (Lambert et al. 2013),

5. Tau processing:
CASS4 (Lambert et al. 2013), FERMT2 (Lambert et al. 2013)

6. Hippocampal synaptic function/cell migration:
PTK2B (Lambert et al. 2013), MEF2C (Lambert et al. 2013)

7. Cytoskeletal and axonal transport:
CASS4 (Lambert et al. 2013), CELF1 (Lambert et al. 2013), NME8 (Lambert et al. 2013)

8. Iris /neuronal development/sodium/patassium/calcium signalling:
SLC24A4/RIN3 (Kingwell 2013)

Table 4: Recent common risk variants and their cellular functions and pathways

6 Treatments

6.1 Pre-Symptomatic treatment & clinical trials

A multi-target therapeutic approach applied in the pre-symptomatic phase of AD might be most effective. of the main targets currently being explored. However, none of the main targets have been validated in phase III trials yet (Figure 9).

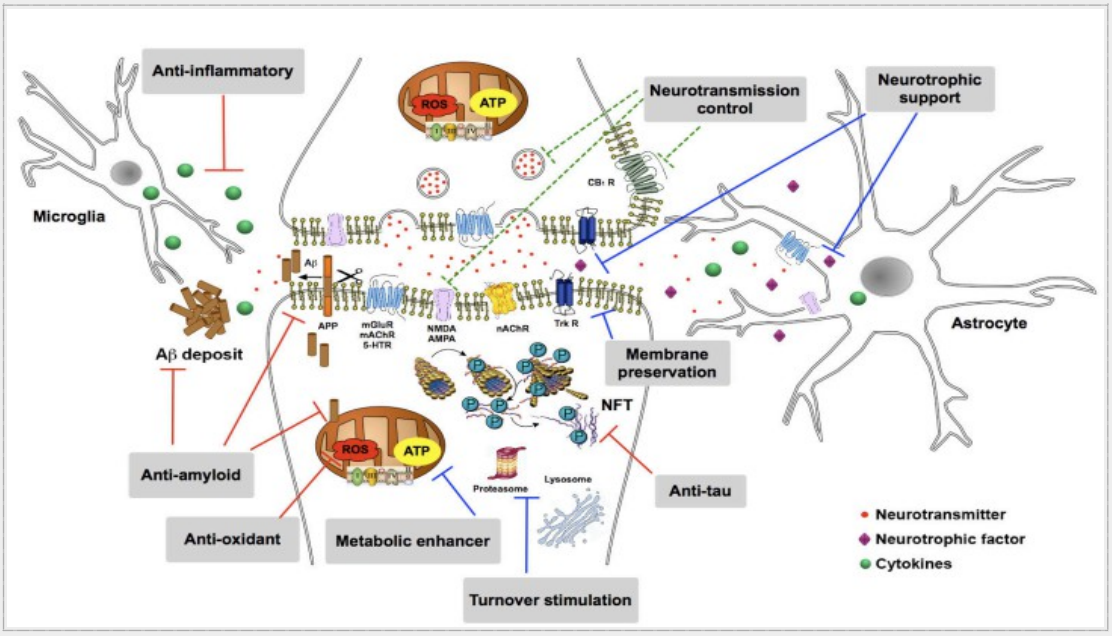

Figure 9: Overview of main cellular AD targets (Adapted from Aso & Ferrer 2013)

The main targets include: (1) A*β* production, (2) tau inhibition, (3) limiting the effects of oxidative stress, (4) controlling neuroinflammation, (5) targeting impaired function of energy metabolism, (6) neurotransmitter dysfunction, (7) synaptic dysfunction, (9) neurotrophic factors, and degradation pathways such as (10) autophagy, and (11) the ubiquitin-proteasome system (Aso & Ferrer 2013).

(1) Aβ production

A*β* production could be regulated by (a) *α*-secretase activators, however, not enough is known about the effect of activating *α*-secretase on other substrates; (b) *β*-secretase inhibitors, nevertheless, adverse effects from BACE1 inhibition can be expected; (c) *γ*-secretase inhibitors, a recent drawback: two large phase III clinical trials of Semagacestat failed because of detrimental cognitive and functional effects of the drug;

(d) Aβ degrading enzymes, there are potential therapeutic properties in insulin-degrading enzymes; (e) decreasing Aβ aggregation, there has been shown indeed significant cognitive improvement in AD patients in a recent phase IIa clinical trial. (f) facilitating Aβ clearance, it has been demonstrated that immunotherapy against Aβ reduced the number of Aβ plaques and the number of dystrophic neurites (Aso & Ferrer 2013).

(2) Tau inhibition

There are four possible scenarios for tau inhibition: (a) preventing tau aggregation; (b) reduction of tau hyperphosphorylation: the neuronal microtubule-associated Tau protein is highly phosphorylated by glycogen synthase kinase 3 (GSK3) which regulates tau binding to microtubules, tau degradation and tau aggregation (Hernández & Avila 2008). In AD, it has been suggested that the peptide β amyloid promotes GSK3 activation, resulting in tau phosphorylation. Thus, targeting GSK3 dysregulation and reduction of tau hyperphosphorylation could be beneficial: for instance, the kinase GSK-3β acts on multiple metabolic pathways, learning and memory, and oxidative stress in mice (Wang et al. 2007; Farr et al. 2014); (c) reduction of tau levels via passive immunization: a study has shown reduced behavioural impairment and tau pathology in two transgenic models (Chai et al. 2011); (d) stabilization of microtubules: A synthetic peptide called 'NAP' has stabilized microtubules in a phase II clinical trial (Aso & Ferrer 2013). However, the treatment effects have not been sufficiently tested.

(3) Limiting the effects of oxidative stress

Oxidative stress can lead to unfolded protein in the endoplasmic reticulum, and cell death through activation of CHOP, caspase-4, and caspase-12 (Aso & Ferrer 2013).

There is a potential beneficial treatment effect with antioxidant compounds: (a) naturally-occurring anti-oxidants, for instance phytochemicals such as, catechins, resveratrols have anti-inflammatory, anti-carcinogenic and anti-infectious properties; (b) mitochondrial antioxidants: free radical damage to mitochondria can occur under exposure to polluted air, radiation or can be induced by chemicals. Nutritional anti-oxidants relieving oxidative stress, inflammation, Aβ levels have shown benefits (Lee et al. 2010).

(4) Neuroinflammation

(Neuro-)inflammation, due to a pathogenic insult, is usually a part of healing but maintained chronically the effects are malicious, and can be observed in AD. Anti-inflammatory drugs have shown positive effects in mouse models, it has been speculated that also naturally occurring anti-inflammatory agents found in nutrition could influence the level of inflammation (Aso & Ferrer 2013).

(5) Energy metabolism

Energy metabolism is altered in the AD brain. Targeting energetic failure by facilitating energy metabolism and availability by regulating the lack of glucose (metabolic deficiency) and oxygen (mitochondrial impairment) could have potential therapeutic effects (Aso & Ferrer 2013).

(6) Neurotransmitter dysfunction

Cognitive and neuropsychiatric symptoms in AD can ameliorate when stabilising the (a) glutamatergic-, (b) serotonergic-, and (c) cholinergic- neurotransmitter systems (Aso &

Ferrer 2013).

(7) Synaptic dysfunction

Stabilising imbalances in lipid function early enough in the disease could reverse cognitive deterioration due to its regulatory effect on synapses (Aso & Ferrer 2013).

(8) Neurotrophic factors and hormones

Many different growth factors and other signalling molecules have been linked to AD (Gold et al. 2010). In particular, the levels of Brain-Derived Neurotrophic Factor (BDNF) are low in AD patients (Lee et al. 2005; Sudduth et al. 2013) and infusion of BDNF improves cognitive functions in ageing primates (Peng et al. 2005). On the other hand, levels of Nerve Growth Factor prohormone (proNGF) are increased in AD, which is accompanied by a downregulation of TrkA (one of the NGF receptors) (Peng et al. 2004; Nagahara et al. 2009). A recent disease model for AD is a mouse line expressing anti-NGF antibodies that presumably impair NGF signalling. Finally, studies have showed that lower plasma levels of the hormone leptin correlate with an increased risk of developing AD (Counts et al. 2004). Although in this latter case, it is still unclear whether leptin levels drop is a cause or a consequence in the etiology of AD. Neurotrophic factors such as BDNF responsible for neuronal survival, differentiation, modulation of dendritic branching and dendritic spine morphology as well as synaptic plasticity, synaptogenesis, and apoptosis should be maintented at certain levels to guarantee their function (Aso & Ferrer 2013). Understanding the impact of the BDNF Val66Met polymorphism in Alzheimer's disease on episodic memory and hippocampal volume could lead to new 'synaptic repair' therapies (Aso & Ferrer 2013).

Neuroprotective hormones, such as estrogen have shown to play an important role in mitochondrial function, neuroinflammation and cognition in AD. Estrogen mediated processes in the IGF1 (insulin-like growth factor 1) pathway and prevented Aβ formation in mice (Aso & Ferrer 2013).

(9) Autophagy

Failure in autophagic processes, due to defective lysosomal acidification (Wolfe et al. 2013), can result in the accumulation of aggregate-prone proteins leading to neurodegenerative processes (Son et al. 2012; Lu et al. 2013).

(10) Ubiquitin-Proteasome System (UPS)

The ubiquitin-proteasome system is a crucial regulatory mechanism in cellular processes such as cell division (Gutierrez & Ronai 2006). The UPS could play a role in early stages of AD, in synaptic dysfunction, and in later stages where neurodegeneration is significant (Upadhya & Hegde 2007). UPS-dysfunction could lead to the accumulation of insoluble protein aggregates. Usually proteins which should be degraded receive a ubiquitin-tag, however if structural changes in the protein substrates (e.g Aβ 1-42) occur, the UPS does not recognize and degrade them. Inhibition of proteasomes thus could contribute to neuropathogenesis (Lam et al. 2000), and targeting UPS components has been suggested as AD treatment.

6.2 Symptomatic treatment

(1) Cognitive

Cognitive rehabilitation could relieve AD symptoms associated with cognitive functioning and improve the performance of the patient in activities of daily living. However, the effect of cognitive training has not yet been clearly defined but it has been noticed that it can improve patients' quality of life (Aso & Ferrer 2013).

(2) Non-cognitive

AD is also accompanied by aberrant levels of several neurotransmitters in neuronal populations such as acetylcholine, glutamate, norepinephrine, serotonin, and somatostatin. In particular, if there are brain lesions in regions associated to cholinergic pathways, AD patients are more severely affected with regard to cognitive and physical performance (Kurz et al. 2011). Glutamate is the main excitatory neurotransmitter in the nervous system, and disruptions in glutamate metabolism have also severe behavioural effects in AD patients (Francis et al. 1999; Butterfield 2004). Several currently applied AD drugs (such as statins) act on somatostatin levels by inhibiting the enzyme HMG-CoA (3-Hydroxy-3-Methyl-Glutaryl-Acetyl-Coenzym A) reductase, which is a rate-limiting enzyme in the metabolic pathway producing cholesterol and other isoprenoids. These drugs indeed belong to the family of cholesterol-lowering medicines which are often used in patients with heart conditions.

In addition, there are possibly common neuropathological mechanisms behind depression and AD related to neurotransmitters such as serotonine and acetylcholine, hypothalamic-pituitary-adrenal glands and the brain derived neurotrophic factor. Thus,

patients show lower stress resistance, anxiety, and irritability which are a major factors contributing to depression (Francis 2003). Targeting these neurotransmitter imbalances could also relieve the AD symptoms of depression.

Chapter 2. White Matter Hyperintensities (WMH)

White Matter Hyperintensities (WMH) are commonly observed on T2-weighted Magnetic Resonance Images (MRI). Variants of the term WMH include leukoaraiosis, white matter lesions, leukoencephalopathy, ischeamic white matter disease, and white matter changes (Hachinski et al. 1987; Fisher 1991; Hoppe et al. 2013). The exact aetiology of WMH is unknown but they are associated with vascular diseases, stroke, and white matter rarefraction (Hernandez et al. 2013; Wardlaw et al. 2013). Vascular risk factors for elevated WMH burden include hypertension, diabetes mellitus, obesity, hyperlipidemia, and stroke (Schneider et al. 2007; Smith 2010; Wardlaw et al. 2013); the same risk factors are associated with AD (Fazekas et al. 1987; Kivipelto 2001) (O'Sullivan 2008; Ben-Assayag et al. 2012; Provenzano et al. 2013; Makedonov et al. 2013), lower cognitive performance (Dichgans & Zietemann 2012), more rapid cognitive decline and dementia (Fazekas et al. 1993; Burton et al. 2004) (Brayne et al. 2009; Matthews et al. 2009; Moon et al. 2011), and contribute to the severity of the disease (Pantoni 2008). In vascular or post-stroke dementia the presence of WMH is much higher compared to individuals with Alzheimer's disease (Jokinen et al. 2005). There are various levels of severity of WMH depending on the type of stroke: (a) lacunar - a type of stroke resulting from occlusion of main arteries; (b) non-lacunar stroke - large-artery atherosclerosis, cardioembolism, stroke of other or of undetermined aetiology (Fein et al. 2000; Wardlaw 2005; Debette & Markus 2010).

WMH indicate hypertensive damage (Nyenhuis & Gorelick 1998), possibly caused by arteriosclerotic changes involving penetration of small arteries and arterioles in the brain

(Toyoda 2008). Hypertension has been linked to high mean diffusivity (detected by diffusion tensor imaging), suggesting altered water content (best visible on FLAIR images) associated with WMH development (Smith et al. 2009; Fazekas & Wardlaw 2013). High diffusivity is linked to increased arterial stiffness, a factor possibly contributing to the pathogenesis of WMH, in particular periventricular WMH (de Groot et al. 2013).

WMH are more prevalent in ageing individuals (Ohmine et al. 2008; Valdés Hernández et al. 2013) while younger people usually do not have severe WMH (Enzinger et al. 2007). Individuals with greater extent of WMH had more severe depression and lower cognitive scores (Scheltens et al. 1992). Elevated levels of WMH have also been found in subjects with multiple sclerosis, Parkinson's disease, bipolar disorder, Binswanger's disease, and a number of demyelinating and bone metabolism diseases (Sundal et al 2012).

1 Imaging methods for WMH detection

WMH can be detected in (1) Structural Magnetic Resonance Imaging (sMRI); (2) Diffusion Tensor Imaging (DTI), and (3) Computer Tomography (CT) brain scans:

(1) sMRI: WMH are best detected by T2-weighted MRI or Fluid Attenuated Inversion Recovery (FLAIR) images in which they can be seen as hyper intensive signals resulting from a more than usual amount of water molecules in particular regions (Heiden et al. 2005). The term 'hyperintensity' is used in T2-weighted MRI images and 'hypointensity' in T1-weighted images.

(2) DTI: The change in apparent diffusion coefficients and fractional anisotropy is

measured to identify WMH (Scheltens & Korf 2000).

(3) CT: WMH changes can also be detected by CT scans in which the high water content is indicated by a low-density (dark-gray) signal (Fazekas et al. 1987). However, CT is usually less accurate than MRI in showing WMH.

2 Regional distribution of WMH in the brain

Previous studies defined WMH as diffuse patchy areas in the white matter, adjacent to basal ganglia or the brain stem, and hyperintense compared to normal white and grey matter on T2-weighted and FLAIR images (Fazekas et al. 2002; Holland et al. 2008). WMH are usually located in periventricular regions but are also found in subcortical areas of the deep white matter extending up to 13 mm beyond the ventricles (Yoshita et al. 2006; Enzinger et al. 2007). Their size ranges from punctate to confluent lesions (Figure 10).

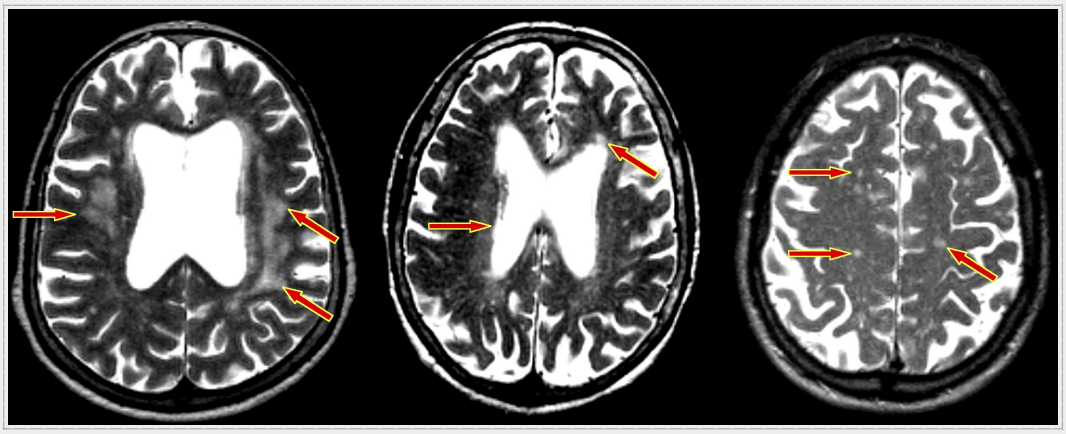

Figure 10: Confluent (left), periventricular lines and caps (middle), and punctate (right) WMH

Several studies have focused on analysing the regional WMH in AD *versus* vascular dementia (Capizzano 2004), and in comparison to normal ageing (Barber et al. 1999; Gootjes et al. 2004). WMH probability maps have shown typical WMH distributions from aged individuals featuring WMH clusters around the anterior and posterior horns of the lateral ventricles and in the centrum semiovale (Valdés Hernández et al. 2013). In Alzheimer's disease, increased WMH have been found particularly in the frontal lobes (Valdés Hernández et al. 2010). Confluent deep white matter changes are more likely to be related to cerebrovascular pathology and cognitive decline (Fellgiebel et al. 2008). It is likely that the regional distribution of WMH is associated with certain pathophysiological conditions.

3 Software for WMH extraction and quantification

There are various methods for the assessment of WMH from MRI images such as automatic segmentation (computer analysis), semi-automatic extraction (computer based with manual removal of false positives) or visual rating scales (Fazekas et al. 2002; de Leeuw et al. 2005; Hernández et al. 2010). The benefit of automatic MRI image processing is that large amounts of data can be analysed in minimal time. Visual rating scales rely on the judgement of the human observer but are necessary for random inspection. Fully quantitative methods often detect higher WMH volume than other methods but the measures are more rudimentary (less sensitive, more false positives

such as CSF artefacts are included) (Wahlund et al. 2001). One problem with automatic processing is that usually all signals across the brain, above a certain threshold, are interpreted in contrast to visual inspection where the radiologist confines his search to very specific regions. Semi-automatic WMH quantification software offer a solution enabling easy masking of brain areas for extraction of WMH in relevant regions (Tanabe et al. 1997).

4 Reasons for heterogeneity of WMH measurements

There are several possible reasons for the wide range of WMH measurements: (a) the choice of imaging methods (CT is less reliable than MRI to detect WMH); (b) a reliable automated/semi-quantitative segmentation method is a precondition; (c) subject pool size; (d) different image protocols; (e) non-optimal selection criteria; (f) inclusion or exclusion of confounding variates such as age, total brain volume and risk factors. For instance, studies including AD patients with high cardio-vascular risk, stroke, and vascular dementia lead to measurements of a larger range of WMH.

5 Radiological correlates

The radiological manifestations of WMH can be observed in a number of diseases such as, multiple sclerosis, dementia, large, and small vascular diseases, hypertensive vasculopathy, and cerebral amyloid angiopathy (Tsai et al. 2014). The examination of the brain regions underlying WMH in deceased subjects does not show abnormalities in white matter tissue on pure visual inspection. However, histo-pathological differences with normal brain tissues have been found, possibly indicating a vascular origin (Kelley

2008; Young et al. 2008). Thus, the clinical and cognitive consequences of WMH have been drawn from pathological correlates and observations in epidemiological studies (Matsusue et al. 2006). For instance, evidence for ischemic/arteriosclerotic changes comes from post-mortem tissues (Fazekas et al. 1993; Pantoni 2010). It has been hypothesised that WMH areas derive blood supply primarily from ventriculofugal vessels originating from the subependymal arteries and are particularly vulnerable to injury due to hypoperfusion (Thomas et al. 2002).

6 Clinico-pathological correlates

Various clinico-pathological correlates have been associated with WMH such as ischemic small vessel disease and leukoaraiosis (Rowbotham & Little 1965). Leukoaraiosis is characterised by demyelinisation, thinner axons, or loss of axons, ependymitis granularis (periventricular, symmetric, patchy loss of ependymal lining, anterior and lateral to the frontal horns), proliferation of glial cells (i.e., astrocytes), thickening of blood vessels with age, cavitation (formation of cavities by high pressure) and small vessel infarcts (Brun & Englund 1981; Wardlaw et al. 2013). WMH neuropathology intersects also with vascular dementia, manifesting in multifocal, diffuse lesions, lacunes and microinfarcts, hippocampal sclerosis, multi-infarct encephalopathy, and diffuse post-ischemic lesions. WMH are often found sub-cortically and disrupt brain connectivity affecting the thalamus, frontobasal or limbic systems (Pantoni 2008).

7 Neuro-psychiatric & neuro-psychological correlates

In AD patients, increased WMH have been related to anxiety, aberrant motor behaviour,

night time disturbance, and apathy (Jellinger 2008). WMH have been found to be correlated with an overall decrease in global cognitive functioning, in particular in processing speed, memory, attention, visuo-spatial ability, motor function, executive functions, daily living activities (Bowen et al. 1990; De Groot et al. 2002; Burton et al. 2004; Capizzano 2004; Murray et al. 2005; DeCarli et al. 2005; van der Flier et al. 2005; Nordahl et al. 2006; Berlow et al. 2010; Moon et al. 2011). In addition, WMH are associated with higher dementia risk (Prins et al. 2004; Smith et al. 2008; Stavitsky et al. 2010). Increased WMH burden is linked to cognitive dysfunction also in non-demented aged individuals (Au et al. 2006; Kim et al. 2008; Debette & Markus 2010). WMH are significantly related to cognitive impairment in AD, however there has been debate on whether cortical, medial temporal lobe or hippocampal atrophy might be a more striking cause for cognitive dysfunction (Son et al. 2012).

8 Molecular correlates & genetic contribution to WMH

The combined analysis of WMH and the level of amyloid deposition could give clues to the underlying mechanisms of the disease (Hirono et al. 2000; Chao et al. 2013). The association of WMH with the AD risk genotype *APOE* has been a matter of debate. WMH are highly heritable and have been linked to small artery ischemic stroke, and therefore could shed more light on the genetics of Small Vessel Disease (Paternoster et al. 2009; Smith et al. 2009). Previous studies have attempted to find associations between polymorphisms in several candidate genes and WMH. Genes involved in lipid metabolism, vascular factors, or blood pressure regulation have been identified but no polymorphisms have shown sufficiently strong associations with WMH (Paternoster et

al. 2009). A recent GWAS has identified two Single Nucleotide Polymorphisms (SNPs) associated with a variation in FBF1 expression in the brain and TRIM47 in lymphoblastoid cell lines; in addition, the functional polymorphisms in TRIM65 and WBP2 have also been linked to WMH in small artery ischemic stroke (Fornage et al. 2011). However, to date, few genetic loci have been identified; it is clear that larger studies need to confirm these associations and ideally find new ones.

Chapter 3. Biomarkers

A biomarker is an objective, quantifiable measurement of the physical or mental state of a patient, or a test subject in a drug development trial, that can be used to diagnose or predict a physiological or pathological condition (Humpel 2011). Biomarkers are classified by usage: antecedent (risk), screening (detection), diagnostic (identification), prognostic (course of disease), stratification (likelihood of drug response). A biomarker should be chosen to reflect fundamental disease features and be specific compared with other disorders. In addition, the measurements have to be reproducible, repeatable, reliable and not harmful. Finally, a method which is non-invasive and easy to perform is preferable - for obvious reasons, non-invasive biomarkers are preferred by patients, especially if the exam has to be repeated. In the case of AD a biomarker for early diagnosis or disease progression is particularly important to enable adequate patient stratification helping to determine drug efficacy in clinical trials (Lovestone 2006; Lovestone et al. 2007; Barber 2010).

Biomarkers can be used in at least five different ways (Lovestone et al. 2009):

(1) In research for new drugs to gain understanding of AD pathogenesis, identifying and validating new drug targets in preclinical and early clinical research.

(2) For diagnosis in clinical trials assisting in the identification of the presence of AD in order to enrol those subjects. Currently, the *APOE* genotype is one of the most used biomarkers for AD patient stratification.

(3) For screening, prevention and early treatment: blood-based and cognitive function

markers.

(4) For monitoring disease progression and drug response in clinical trials and adjusting interventions.

(5) For risk prediction and prevention: identifying subjects who most likely respond to a certain treatment.

Since abnormal changes associated with AD occur before there are easily detectable symptoms, such as memory impairment, biomarkers linked to such changes could provide early warning of possible AD.

Biomarkers such as phenotypic measurements (cognitive decline scores, etc.) are often not very accurate because cognitive tests yield inaccurate results based on how the patient is feeling on a certain date, time, season or other biocyclic/hormonal influences. The inclusion of factors contributing to disease such as white matter hyperintensities, age, sex, education, and *APOE* status could improve biomarkers' accuracy. Here, we focus on a biomarker capable of predicting disease progression.

1 Neuroimaging

Advances in imaging technology and biomarkers have allowed earlier detection of brain changes related to AD. Remarkably, these changes appear years before the first traditional symptoms, opening up the possibility of pre-symptomatic trials. Information from brain images of patients and high-risk individuals can potentially become surrogate markers in clinical trials. One advantage of imaging biomarkers is that they are non-

invasive. Alzheimer's disease's changes regarding WMH, atrophy, hippocampal atrophy, large ventricles, can be investigated. The estimated accuracy depends on the measurement target and the imaging method: Single-Photon Emission Computed Tomography (SPECT) has lower accuracy (50-55%) than MRI (80%). Metabolic changes in the brain can be detected with Positron Emission Tomography (PET), blood flow with Functional Magnetic Resonance Imaging (fMRI), and the diffusion process of molecules with DTI- Diffusion Tensor Imaging.

(1) Magnetic Resonance Imaging: In general Magnetic Resonance Imaging (MRI) can create images of many parts of the body providing structural images. An MRI sequence is an ordered combination of radio frequency signals and gradient pulses designed to acquire the image information. The pulse sequences are Fluid Attenuated Inversion Recovery (FLAIR), Spoiled Gradient Echo (SPGR), T2-weighted and T1-weighted or Proton Density (PD). Scanners can have different field strengths, with later models having field strengths up to 3 Tesla. The acquisition parameters are depicted by matrix size, field of view, slice thickness and 2D or 3D acquisition: frequency/phase encoding. Image contrasts can be T1 weighted or T2 weighted with tissue parameters T1, T2 and acquisition parameters Repetition Time (TR), Echo Time (TE). In MRI the spinning nuclei align with the direction of the magnetic field. For example, water nuclei resonate at 64MHz in a 1.5 Tesla field. The advantage of MRI imaging is that the reliability and reproducibility of these scans is high (Lovestone et al. 2013). Common AD related MRI measures are: whole brain atrophy, white matter hyperintensities (Hampel et al. 2010), hippocampal atrophy (Brickman et al. 2008), and enlargement of ventricles (Henneman

et al. 2009).

(2) Functional Magnetic Resonance Imaging (fMRI) is a commonly utilized research tool to detect functional differences (blood flow) in the brain compared to healthy controls and may be a promising biomarker for AD (Damoiseaux 2012). fMRI studies have detected increased brain activation in patients treated with acetylcholinesterase inhibitors in MCI and AD (Nestor et al. 2008) (Saykin et al. 2004).

(3) In Positron Emission Tomography (PET) imaging a radioactive tracer shows the amyloid plaques in the living human with up to 75% accuracy. Using specific tracers such as N-methyl-[11C]2-(4′-methylaminophenyl)-6-hydroxybenzothiazole, also known as Pittsburgh Compound-B (PiB), amyloidosis can be quantified. PiB-PET studies revealed significant PiB retention in AD patients compared to control individuals (Rombouts 2002).

2 Cerebrospinal fluid

Cerebrospinal fluid (CSF) biomarkers are currently the most efficient for diagnosis of probable AD, for predicting conversion from MCI to AD in the prodomal phase (Mattsson et al. 2009), and for predicting the rate of cognitive decline in AD (Snider et al. 2009). Several core biomarkers have been established Aβ40, Aβ42, total tau (t-tau), and phosphorylated tau (p-tau) which indicate the amyloid levels in the brain and neuro-fibrillary pathology (Hansson et al. 2010). In AD patients, there is a significant drop in the concentration of Aβ(1-42) peptides in the cerebrospinal fluid that likely reflects the accumulation (deposition) of the toxic protein fragment in the brain, whereas total tau is elevated in CSF (Wu et al. 2012).

Aβ(1-42), t-tau, and p-tau demonstrated a much higher accuracy (95%) in the diagnosis of AD than the combined groups of MCI and healthy controls (Hampel et al. 2010). The CSF is extracted from the spine of the patient via lumbar puncture. The accuracy is usually relative good with 80-90% sensitivity and specificity. The disadvantage of the method is that the procedure for obtaining CSF is invasive; therefore, less preferred by patients for repeated measurements, and in addition, in some countries, it is not approved for routine use. The basic CSF biomarkers reflect pathological processes such as inflammation, Brain Blood Barrier function, or intrathecal immunoglobulin production (Hampel et al. 2010) (Blennow et al. 2010).

3 Blood

On the one hand, the use of blood as a biomarker for Alzheimer's disease is challenging.

On the other hand, measurements of blood are easy to obtain, even at the patient's home, inexpensive, and repeatable. This offers also a good possibility for already frail, elderly people. The assumption is that if AD can be diagnosed earlier in the disease stage it can be treated better, however at the moment this is not proven in real life and more importantly not with medications. There is communication between the blood and the brain but they are not directly connected (blood-brain-barrier). Thus, if changes were found in blood careful validation of the new potential biomarker is necessary. Blood plasma proteins are potential candidates for identifying aged individuals at risk for Alzheimer's disease, diagnosis of AD, and could possibly support in the prediction of decline in Alzheimer's disease. Evidence has suggested that there are protein signatures of pathological processes in blood before disease onset; this evidence has been drawn from candidate protein studies or inflammatory markers (Leung et al. 2013; Kiddle et al. 2014).

Different signatures such as (1) proteins, (2) RNA, (3) antibodies, and (4) lipids can be found in blood and serve as biomarkers in the future:

(1) Proteins can be detected in plasma, serum, or other cellular compartments. They have a number of advantages: (a) respond to changes in health status and drug administration; (b) are readily accessible via the blood circulation; (c) measuring multiple proteins simultaneously can capture the contribution of various – and sometimes unforeseen – biological processes; (d) new high-throughput technologies enable rapid progress in the search for protein biomarkers (Thambisetty & Lovestone 2010). However, a disadvantage can be that proteins reflect not only the internal but also

external environment. For instance, a certain degree of disease could be associated with life-style and protein signatures reflecting this life-style could be found rather than reflecting the disease itself.

(2) RNA can potentially contain information useful as a biomarker for AD diagnosis in early stages (Lunnon et al. 2013; Kumar et al. 2013).

(3) Reddy *et al.* have recently identified diagnostically useful antibodies (IgGs) by using natural or synthetic molecules that retained significantly more IgGs in patient samples than in controls. In this way, they have found two potential IgG biomarkers for AD (Reddy et al. 2011).

(4) A very recent study has determined lipids in blood from cognitively normal older individuals which predicted conversion to MCI or Alzheimer's disease within a 2–3 year period with over 90% accuracy (Mapstone et al. 2014).

4 Cognitive Assessments

For assessing the disease severity usually a battery of cognitive assessments is available. The advantage of using cognitive measures as biomarkers is that these methods are not invasive and provide good accuracy ranging between 60 – 70%. Frequently, methods such as the Minimal Mental State Exam (Folstein et al. 1975), DRS (Mattis 1976), ADAS (Rosen et al. 1984), CAMCOG (Huppert et al. 1995), and Hodkinson Mental (Hodkinson 1972) are utilized to assess cognitive performance. The presence of AD can be detected with analysis of the pattern and evolution of cognitive deficits, delineation of

the particular cognitive strengths and weaknesses of individual patients, and monitoring disease progression and the effects of treatment. Moreover, the method is relatively cost-effective. Apart from adequately trained medical staff and a recent version of the assessment questionnaire, no major accessories or equipment are needed.

Chapter 4. Aims & Objectives of the Thesis

This work aims to contribute to a better understanding of disease progression in AD. Molecular, cognitive and structural brain changes linked to the disease will be identified and related to AD progression. Finding molecular signatures in blood can reveal important relationships within the implicated panels of mRNAs and proteins. Once validated, the signatures can be incorporated in the development of a blood test for AD and ideally qualify as an endpoint in clinical trials.

Many promising drugs are currently being tested in clinical trials for AD but their efficacy is difficult to assess using existing outcome measurements such as psychometric scores (L. Zhou et al. 2012; Yu et al. 2013). Biomarkers capable of accurately predicting rates of disease progression are urgently needed: they can provide important information on the current pathological state of a potential participant in a clinical trial (Lovestone 2006). Novel biomarkers that enable better patient stratification models, with optimized numbers of participants, could significantly reduce the length of trials, and could help to provide a time-frame for preventive measures. Accordingly, the main objectives of the study (1 and 2 below) are focused on investigating factors which could influence disease

progression.

The 1st objective is to evaluate the association between WMH and the rate of cognitive decline in AD. For this purpose, several statistical models of cognitive decline with different covariates are tested. WMH are hypothesized to contribute significantly to AD cognitive decline and to explain a proportion of the variability in the rate of disease progression. Here, the focus was on WMH across the whole brain and disease progression indicated by cognitive decline across the specific cognitive domains measured by MMSE, ADAS-cog, and CDR-SOB.

The 2nd objective is to apply statistical classifiers in order to define a panel of mRNA transcripts and proteins correlated with WMH and cognitive decline in AD.

Part II. DATA AND EXPERIMENTS

Chapter 1. Association of White Matter Hyperintensities with cognitive decline in AD

1 Introduction

1.1 WMH and cognitive decline

A number of studies have established a relationship between WMH and cognitive decline (Yoshita et al. 2006) (Kelley 2008) (Kearney-Schwartz et al. 2009) (Weinstein et al. 2013) (Jacobs et al. 2012) (Wardlaw et al. 2013). In the past there were inconclusive results regarding the clinical significance of WMH, specifically in AD (DeCarli et al. 2004) (Smith et al. 2008). However, later studies increasingly recognised WMH to be associated with AD-cognitive decline (Carmichael et al. 2010) (Moon et al. 2011) (Gold et al. 2012). Patients who have co-existing AD and WMH experience more severe longitudinal cognitive decline than those having either alone (Yoshita et al. 2006). Thus, there is a clear need for investigating the effects of WMH on cognitive decline in AD.

WMH are signal abnormalities commonly detected on MRI brain scans in ageing individuals and patients with cerebrovascular disease, and have been associated with cognitive loss and AD (Kelley 2008; Valdés Hernández et al. 2012; Wardlaw et al. 2013). The prevailing view of WMH is that they represent perfusion abnormalities, in

particular in the posterior cortex, implicating them in the presentation and pathogenesis of AD with amyloidogenic pathology (Kelley 2008).

WMH could be linked to increased rate of cognitive decline, possibly in combination with other variables such as, advanced age, gender, APOE ε4 genotype, and especially atrophy (van der Flier et al. 2004). For defining an AD disease progression biomarker the best combination of variables still remains to be elucidated. Whole brain atrophy is clearly a major factor influencing cognitive decline (Provenzano et al. 2013; Matthews et al. 2009; Carmichael et al. 2010). Hippocampal atrophy is a strong predictor for cognitive decline (Schmidt et al. 2005; Brickman et al. 2008; Mok et al. 2011). Age, APOE ε4 genotype, and gender can also influence the rate of disease progression (Ito et al. 2011). Many age-related changes occur in small arteries, arterioles, venules, and capillaries having malicious effects on the brain leading to hypertension, increased WMH and lower cognitive performance (Murray et al. 2005; Kearney-Schwartz et al. 2009; Wardlaw et al. 2013). Carriers of the APOE ε4 allele are at increased risk of developing AD (Schiepers et al. 2012). The effect of APOE on WMH has been debated and remains unclear. Some studies did not find a relationship between APOE ε4 and WMH volume (Kim, 2013). However, APOE has been shown to be correlated with WMH and cognitive decline in other studies (Pantoni 2010; Salmon et al. 2013). An association of APOE ε4 with the rate of cognitive decline in healthy, aged individuals has been found: non-demented carriers of the APOE ε4 allele are more likely to develop AD and have increased rate of cognitive decline (Schiepers et al. 2012).

In the past, there has been mixed evidence regarding the importance of gender regarding the risk of AD (Hebert 2001; Barnes et al. 2003; Wen & Sachdev 2004). However, recent studies have identified gender as an important covariate in disease progression models of AD (Ito, 2011). Higher education has been associated with a higher cognitive reserve, lower CDR sum of boxes scores and significantly modified the association of WMH with cognitive abilities (Scarmeas et al. 2006).

Short-term measurements of WMH-associated AD-cognitive decline might be important in clinical trials to determine the efficacy of a drug or in a situation in which the care situation of a patient has to be decided, such as admission to a nursing home. WMH might be included as a covariate in statistical prediction models to better define clinical endpoints.

Clearly, future work should focus on investigating the cause-effect relationships between WMH and cognitive decline, in order to better predict AD progression. Currently, it is hypothesized that WMH are important radiological correlates of cognitive functioning, however studies remain inconsistent in reporting the actual effect size (Kelley 2008). Heterogeneous AD pathology is complicating these efforts.

1.2 Aims

The hypothesis under investigation was: "*WMH associate with more rapid cognitive decline in AD, and their inclusion in a prognostic biomarker of AD progression might increase its prediction accuracy*".

To test this hypothesis, linear mixed models were used, including individuals with AD who were assessed over the course of approximately 1 year, with three different neuropsychological assessments, CDR-SOB, MMSE, and ADAS-cog. The cognitive change in assessment scores, under consideration of MRI measures of WMH volumes from the 1st visit, and other covariates, was evaluated.

2 Methods

2.1 Subjects

The AddNeuroMed cohorts

The subjects were drawn from the AddNeuroMed study which had 781 participants. Blood samples were collected from each subject for DNA (*APOE* genotyping) and RNA analyses (gene expression). Subjects who met MRI inclusion criteria (no claustrophobia, no trauma or surgery which may have left ferromagnetic material in the body, ferromagnetic implants or pace- makers, and the ability to lie still for at least one hour) were invited to undertake an MRI scan until a total of ~20 subjects per diagnostic group (AD, MCI, CTL) per centre were scanned (Lunnon et al. 2013). There were 6 centres (Kupio, Lodz, London, Perugia, Thessaloniki, and Toulouse). In total there were ca. 20x3x6= 360 samples. However, during the sample quality control process a number of samples had to be removed (Lunnon et al. 2014) (Sattlecker et al. 2014). The total number of subjects of blood samples matched with mRNA measures was **331**. The total number of subjects matched with proteins measures was **337** of which 104 AD patients

had baseline MRI scans (Simmons et al. 2011). Four subjects were excluded because they had a glioma or a stroke. WMH from the remaining **100** brain scans were extracted using BRIC1936 software (Valdés Hernández et al. 2012). The longitudinal cognitive assessments were available for AD patients with all five visits: CDR-SOB (n=90), MMSE and ADAS-cog (n=90). The 5th visit took place between 323 days and 1 year 117 days after the 1st visit, thus, we describe the time period with ca. 1 year. The visits took place in approximately 3 months intervals. After matching with WMH with CDR-SOB, MMSE, and ADAS-cog fewer subjects remained: **84** subjects were included in the linear mixed models for the rate of cognitive decline with the covariates WMH, atrophy, and gender (Table 5 & 6).

Total subjects	781	APOE	Gender	Education	Age of onset	Disease duration	Atrophy
a. MRI scans for AD	104						
b. Excluded	4						
c. WMH extracted	**100**	100	100	100	100	100	100
d. CDR-SOB	90	85	87	85	88	88	88
MMSE	90	85	87	85	88	88	88
ADAS-cog	90	85	87	85	88	88	88
(min 5 visits)							
e. WMH+CDR-SOB	**84**	84	86	84	87	87	87
WMH+MMSE	**84**	87	86	83	87	87	87
WMH+ADAS-cog	**84**	84	86	86	87	87	87

Table 5: Number of participants in the AddNeuroMed study, (a) number of AD patients with brain scans, (b) excluded subjects, (c) quantified WMH from AD patients, (d) cognitive measures obtained from 5 visits, (e) number of subjects remaining when cognitive measures matched quantified WMH, used in linear mixed models.

Characteristics of AD subjects with quantified WMH at visit 1 (baseline)	N=100
Age at baseline (years)[a]	74.7 ± 6.4
Age of disease onset (years)[a]	76.5 ± 6.5
Gender female/male	64/36
Education (years) (n=97[a]; 3 unknown)	6 ± 4.3
Disease duration since first diagnosis (months)[a]	48 ± 28.6
Baseline cognitive scores (points) CDR-SOB[a] MMSE[a] ADAS-cog[a]	 6.4 ± 3.0 20.0 ± 4.7 28.0 ± 9.7
Rate of cognitive decline over 1 year (points), calculated with daily rate x 365.24 CDR-SOB n=85[a] MMSE n=83[a] ADAS-cog n=84[a]	 1.5 ± 1.6 -1.4 ± 1.8 1.9 ± 0.5
WMH median [IQR] (mm)[3]	3310 [7777]
Atrophy[a] (normalized by the subject's intracranial volume)	0.82 ± 0.02
APOE *e*33; *e*34; *e*44; unknown	36; 41; 15; 8

Table 6: Subject characteristics of the AddNeuroMed study; [a]Data are represented as mean ± standard deviation.

All available details of the patient cohorts were integrated. Information about vascular risk factors was not collected. It seems that the group who had only 6 years of education was growing up during the Second World War and had indeed had only 6 years of school attendance. They were on average 75 years old this would be during the 1940s.

The AD patients of the AddNeuroMed cohorts receive Donepizil, Rivastigimine, and Galantamine which could explain that their average decline is not as steep as in untreated individuals with AD. The fact that the age of MRI scan was lower on average than the average age at AD diagnosis is possible because samples from subjects who had undergone an MRI scan and had subsequently changed their diagnosis from MCI to AD within 2 years of the sample being analysed were included in the studies (Lunnon et al. 2014).

The Dementia Case Register cohort

For the King's Health Partners Dementia Case Register (Kiddle et al. 2013) cohort 47 baseline scans were available of which 3 had to be excluded, WMH was extracted from 44 MRI volumes (Table 7).

Dementia Case Register (DCR)	*Number of subjects*
Available AD baseline scans	47
Excluded: 3 had a stroke	3
WMH quantification	44

Table 7: Number of AD subjects with MRI and WMH quantification in the DCR cohort

After matching the **44** WMH volumes only **12** subjects remained who had also longitudinal cognitive measures (Table 8). Therefore, the cohort could not be used in the linear mixed model for cognitive decline. However, in the second study of the current work in which WMH was analysed together with proteins, the 44 WMH volumes could be included (Table 19). Below a summary of the DCR subject characteristics (Table 9).

Cognitive assessment (min 3 visits)	Total	APOE	Gender	Education	Age of onset	WMH
WMH	**44**					
MMSE	30	29	29	28	29	**12**
ADAS-cog	30	30	30	30	30	**12**

Table 8: Number of participants from the Dementia Case Register study

Characteristics of DCR subjects with quantified WMH at baseline	*N=44*
Age at baseline (years)[a]	73.5 ± 14.9
Gender female, male	18, 26
WMH (median [IQR]) in mm^3	4871 [10954]

Table 9: Subject characteristics of the DCR study: [a]Data are represented as mean ± standard deviation.

2.2 Cognitive assessments and calculation of the rate of cognitive decline

The rate of cognitive decline in AD patients was calculated using MMSE, ADAS-cog, and CDR-SOB, for subjects of which longitudinal assessments were available from 5 visits within a one year period. The rate of decline was estimated by fitting separate mixed models for each assessment scale. The cognitive performance of AD patients was assessed at five visits in a three month interval over the course of 1 year. The package 'nlme' in R was used to generate the statistical models. The average baseline cognitive outcome and average change in cognitive outcome over follow-up time was calculated for all patients as a group (random effects) and subject-specific intercept and slope which reflected the deviation from the group average (fixed effects) including the adjustment for covariates such as, WMH, atrophy, age a baseline, disease duration, gender, education, and *APOE* genotype, were calculated (advanced model). Follow-up time was defined as the number of days passed since the baseline visit, and 5 timepoints, 3-months apart were recorded for each patient. Covariates which were significant (P<0.1) in the advanced models of CDR-SOB, MMSE, and ADAS-cog, and remained significant in the simple model, were kept in the final model (WMH, atrophy, and gender). The coefficients (β-value) for the follow-up variable (days) denoted the slope (per day) and the coefficients (β-value) for each variable indicated their association with overall cognitive levels. Power and sample size were calculated using a R-script (see appendix) provided by Donohue and colleagues (Donohue et al. 2013).

2.3 Structural magnetic resonance imaging

MR Image parameters

MRI images were available from a subset of the AddNeuroMed subjects (Simmons et al. 2011). The image data were acquired using six different 1.5 T MR systems (four General Electric, one Siemens and one Picker) and a quadrature birdcage coil for RF transmission and reception. The acquisition process was designed to be compatible with the Alzheimer disease neuroimaging initiative (ADNI). A high resolution saggital 3D MP-RAGE dataset, using a three-plane localizer, and an axial proton density/T2-weighted dual echo fast spin echo dataset was acquired. London cohort for AddNeuroMed were acquired at King's College London with the following parameters: DESPOT1 pulse sequence for T1 mapping with TR¼8.45 ms, TE¼2.92 ms, one data average and voxel size 1.2*1.02*1.02 mm, and DESPOT2 pulse sequence for T2 mapping with TR¼3.6 ms, TE¼1.8 ms, one data average and voxel size 1.2 x 1.02 x 1.02 mm. The Quality Control (QC) took place immediately after acquisition at each site and an on-site radiologist excluded any subjects with non-AD related brain pathologies (Simmons et al. 2011). Firstly, regular phantom scans were performed at each site using the ADNI phantom (manufactured from urethane which consists of an array of spheres arranged in a precise geometric pattern). Quantitative measures of signal to noise ratio, uniformity and geometric distortion were recorded and assessed using the ImageOwl web-based automated QC system (www.imageowl.com). Secondly, in vivo measures were validated using two young adult volunteers (to ensure no age-related changes) who were periodically scanned after any hardware or software upgrade. Human phantom data

were processed using an automated image analysis pipeline (Jack et al. 2008). The *in-vivo* measures QC included: onsite visual image control according to the QC protocol, image data transfer, data upload and automated QC, manual QC at the Data Control Centre, feedback and real time statistics. Trained experts performed the on-site visual image control and manual QC to assess technical aspects such as full brain coverage, wrap-around artefacts, motion artefacts, intensity inhomogeneity. Preprocessing, co-registration and optimization has been previously performed within the group: The AddNeuroMed images were transformed from DICOM to a sagittal T1 and an axial, co-registered pair of PD/T2 images. For the DCR images three modalities were received as a co-registered axial set T1/T2/FLAIR. The spatial orientations, voxel size, and number of slices were compatible across the six study sites, with some exceptions: some images were received with different properties (inverted orientation, weak contrast) from which some but not all could be co-registered and normalised using spm8 and ImageJ.

MRI Subject criteria

Inclusion criteria for Alzheimer's Disease subjects

(1) AD subjects were diagnosed using the NINCDS-ADRDA (McKhann et al. 1984), and Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria for possible or probable AD (DSM–IV–TR (2000) 4th ed., text rev.), following a semi-structured interview with the patient and an informant and detailed case history;

(2) subjects aged 65 years or above.

Exclusion criteria for Alzheimer's Disease subjects

(1) significant neurological or psychiatric illness other than AD; (2) significant

systematic illness or organ failure.

Structural MRI plays a role in defining entry criteria for AD clinical trials and as an outcome measure of AD disease progression (Kennedy, 2009). For each study, the most appropriate protocols should be chosen. In the AddNeuromed, the range of protocols included a high-quality high-resolution T1-weighted 3D sequence guaranteeing accurate morphometric information as well as dual contrast T2-weighted/proton density information to a certain AD brain pathology.

T2-weighted MR sequences are much more sensitive than CT for the detection of WMH (Gauthier, 2006). T2-W1 has high sensitivity for thalamic lesions. It is recommendable to include several T2-weighted sequences such as T2 and FLAIR, for instance FLAIR has the advantage of detecting cystic lesions. In addition, a combination of T1-W1 and FLAIR could identify more severe lesions. It is to be noted that very severe lesions are associated with cognitive impairment and cognitive decline (Gauthier, 2006). FLAIR images have not been acquired in the AddNeuroMed study but could have given additional information on WMH load and morphometry, and AD brain pathology.

2.4 WMH extraction methodology

The first approach explored was based on an automated procedure for the assessment of WMH by multispectral (T1, T2, PD) MRI (Maillard et al. 2008). This procedure could not successfully be applied because the T1 image data of most subjects were not suited to this approach without implementing time-consuming extensions. The second

approach involved a semi-automatic software: BRIC 1936 - Brain Research Imaging Centre of The University of Edinburgh (Valdés Hernández et al. 2012). Currently, there is no fully-automated WMH extraction method because removing misclassified WMH regions remains an issue. BRIC forms part of MCMxxxVI (Multispectral Colour Modulation and Variance Identification), a suite of image manipulation and analysis tools written in MATLAB by Dr. Maria Hernandez, ported to C++ by the The Software Sustainability Institute. Using the BRIC software suite, quantification of WMH was successfully performed. Image fusion (of different image modalities), WMH extraction (based on RGB-values), and removal of false positives is performed with the in BRIC integrated tools. The output consists of descriptive statistics and image data with the file-type ANALYZE (.img/.hdr). Blinded to clinical information, WMH were defined, as described in previous studies: diffuse patchy areas in the white matter, basal ganglia or brain stem, and hyper-intense compared to normal white and grey matter on T2-weighted and FLAIR images. WMH probability maps shown in previous studies were visually assessed to prepare for the WMH extraction (Enzinger et al. 2006). Tissue segmentation in BRIC relies on defining a set of Red-Green-Blue (R-G-B) values (Table 10). To determine the best and most cohort specific thresholds, the first image of the cohort was used as a reference and the best WMH threshold chosen on visual assessment. Once the best combination for differentiating WMH from other brain tissue was found, the colour parameters were applied to the whole cohort.
In total we had 6 specific combinations of R-G-B parameters from the AddNeuroMed study of which the images were extracted from T2 alone (Figure 11). For the DCR cohort WMH have been extracted from T1/ T2 and FLAIR (Figure 12).

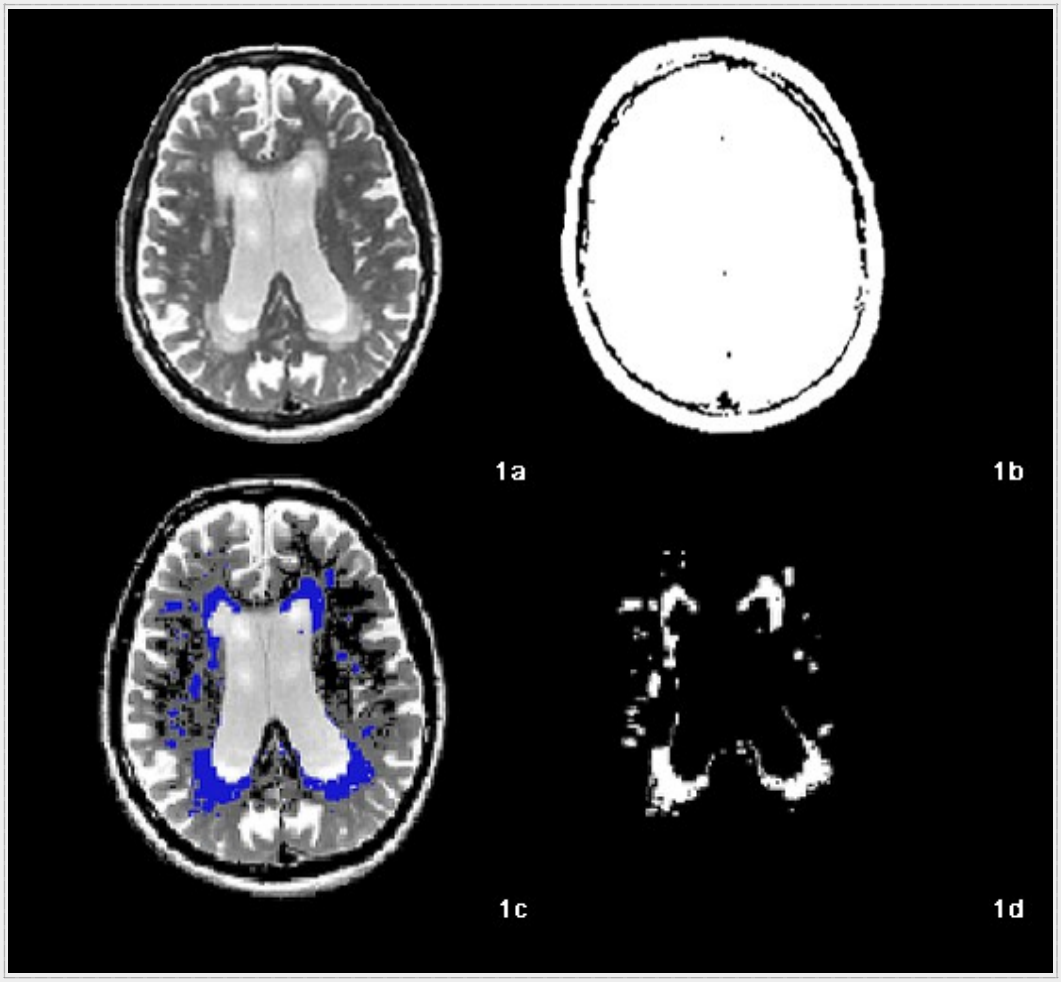

Figure 11: Example of WMH extraction from T2-MRI for the AddNeuroMed cohorts; slice from an AddNeuroMed subject with extensive caps of WMH and punctuate lesions extracted with BRIC: (1a) T2 MRI volume; (1b) brainmask; (1c) WMH in blue overlay on a T2 volume; and (1d) segmented WMH.

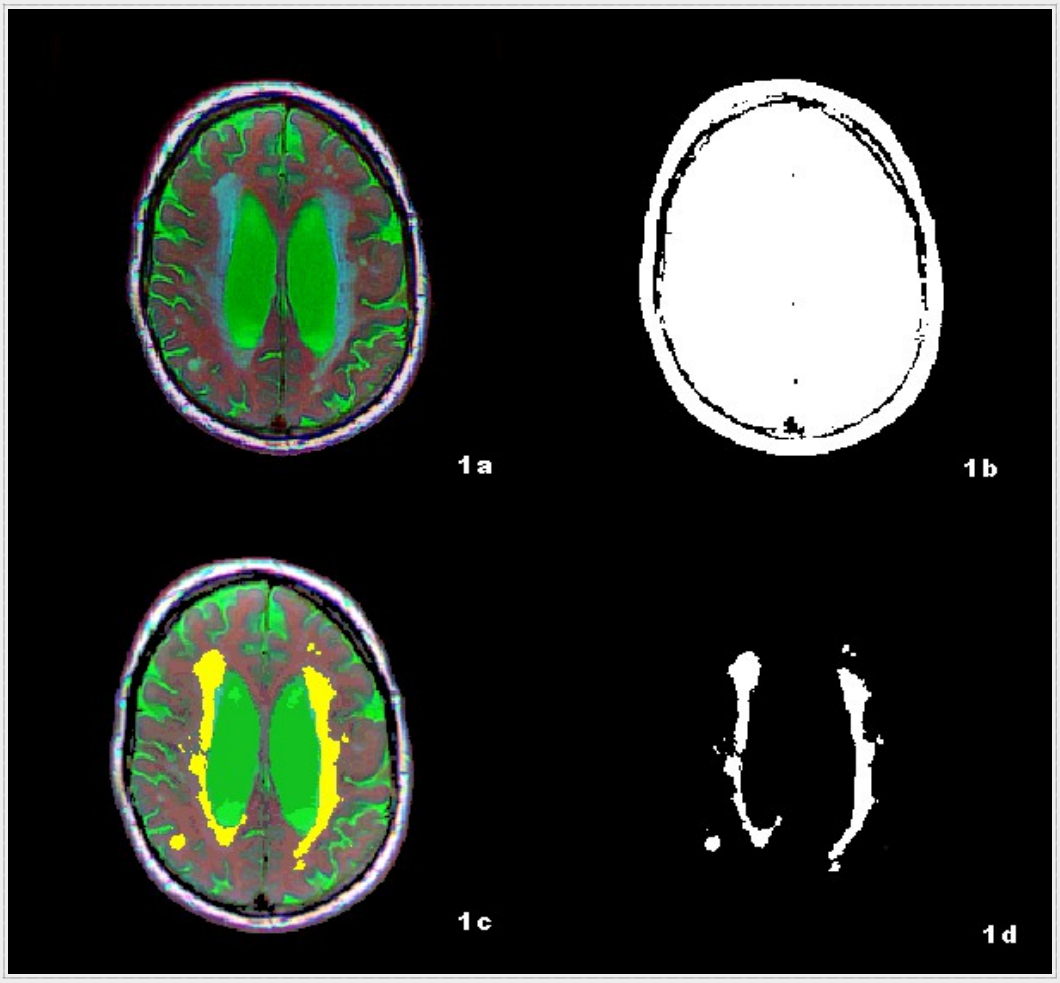

Figure 12: Example of WMH extraction from the Dementia Case Register cohort: slice from a DCR subject with extensive periventricular WMH extracted with BRIC: (1a) multi-spectral Colour Fusion of T1/T2/Flair MRI volumes; (1b) brain tissue mask created with ImageJ; (1c) segmentation of WMH volume in yellow showing on the overlay on a R-G-B colour-fused image; and (1d) segmented WMH.

MRI image acquisition and preprocessing was compatible (same parameters) with the AddNeuroMed study (Simmons et al. 2011). The WMH extraction was different to the AddNeuroMed study, WMH was quantified using co-registered sets of T1, T2, and FLAIR images (Table 10). Areas in which WMH was incorrectly detected (false positives) were removed manually with the BRIC software by masking and deleting relevant foci and regions, and their WMH extracted.

WMH extraction from T2 MRI	*Red / green / blue parameters (% range of the full scale [0-265])*
1. AddNeuroMed/ Kupio	58%-86% – 58%-86% – 58%-86%
2. AddNeuroMed/ Lodz	51%-70% – 51%-70% – 51%-70%
3. AddNeuroMed/ London	70%-86% – 70%-86% – 70%-86%
4. AddNeuroMed/ Perugia	64%–90% – 64%–90% – 64%-90%
5. AddNeuroMed/ Thessaloniki	59%-86% – 59%-86% – 59%-86%
6. AddNeuroMed/ Toulouse	58%-90% – 58%-90% – 58%-90%
WMH extraction from T1, T2, and FLAIR	
Dementia Case Register	8%-74% – 74%-84% – 7%-12%

Table 10: WMH extraction parameters for the six AddNeuroMed cohorts and the Dementia Case Register

The degree of agreement among WMH lesion extraction with BRIC and Fazekas validation on visual inspection of the T2 images is high ($n=89$, $R^2= 0.66$, $p<0.001$).

2.5 Atrophy measures

Whole brain volumes were obtained using FreeSurfer 5.1.0 from 274 AddNeuroMed subjects who had undergone structural magnetic resonance imaging (sMRI). The extraction of atrophy measures was not in the scope of this thesis; detailed information about data acquisition, pre-processing, and quality control assessment have been

described in detail elsewhere (Simmons et al. 2009) (Simmons et al. 2011) (Westman et al. 2011).

2.6 Validation of WMH

The validation was performed by the author of this thesis and an independent observer according to Fazekas scale (Fazekas et al. 2002). Different WMH appearances were classified as: absent (0); punctuate, thin periventricular lines, small lesion caps (1); early confluent and thicker periventricular lines, large lesion caps (2); and confluent with punctuate, lesion caps and lines (3). In particular, attention was paid to false positives (Fazekas et al. 2002). Some hyper-intense regions have been shown to represent normal anatomical variants such as symmetrical hyper intense "caps" around the frontal horns, regular periventricular lines, signal alterations in the basal ganglia, pons, and in particular Virchow-Robin spaces or lacunas (Enzinger et al. 2007). The WMH volumes were significantly correlated with the Fazekas scale (n=100, $P<0.05$).

3 Results

3.1 Baseline WMH as a predictor of cognitive decline in AD

The main objective was to evaluate relationships between MRI measures of WMH volumes from the whole brain, measured at the first visit, and the rate of cognitive disease progression, calculated from repeated visits over a period of ca. 1 year, after the

MRI scan. Mixed-effects models evaluated relationships between WMH volume and changes in outcome measures including MMSE, ADAS-cog, and CDR-SOB.

Higher baseline WMH volumes were found to be significantly associated with greater cognitive decline in AD ($P<0.05$) with three assessments (CDR-SOB, MMSE, and ADAS-cog). Specifically, each 1-*Standard Deviation(SD)* increase in baseline WMH was associated with an additional 0.0001-*SD* increase per day (x 365.24 = 0.04-*SD* increase per year) in CDR-SOB, with an additional -0.0002-*SD* decrease per day (x 365.24 = 0.07-*SD* decrease per year) in MMSE, and with an additional 0.0003-*SD* increase per day (x 365.24 = 0.11-*SD* increase per year) in the ADAS-cog (Table 11).

Significant Association of Baseline WMH with Change in Cognition in the CDR-SOB, MMSE and ADAS-cog model									
Variable	CDR-SOB n=84			MMSE n=84			ADAS-cog n=84		
	β	*Std. Error*	*P* Value	β	*Std. Error*	*P* Value	β	*Std. Error*	*P* Value
Baseline WMH	0.0001	0.00004	<0.05*	-0.0002	0.00007	0.02*	0.0003	0.00015	0.04*
Atrophy	-39..47	8.9184	<0.001*	30.99	14.613	0.04*	-45.7	31.3787	0.1^
Gender (Male)	-2.11	0.71	<0.05*	2.43	1.163	0.04*	-4.85	2.4984	0.06*

Table 11: Summary of the CDR-SOB, MMSE, and ADAS-cog mixed-effects models of cognitive change using baseline WMH volume and the predictors atrophy, education, and gender as fixed effects. The regression coefficient is shown for the fixed effect followed by the associated P value ("" denotes that the P-value is significant at the 0.05 level, ^ denotes that the P-value is significant at the 0.1 level), and standard errors of coefficients are reported. Regression coefficients represent the number of standard deviations' difference in the cognitive baseline that are associated with a 1-unit change in a predictor measured at baseline. The corresponding protocol of the R output can be found in Table 47-51.*

The relationships between WMH and cognition were marginally modified when *APOE* genotype, age, education, and disease duration were added to the models. In particular baseline WMH volume, in the ADAS-cog model, was then only significant at P=0.1 level (Table 12).

Association of Baseline WMH with Change in Cognition including covariates education, *APOE*, gender, onset, disease duration, atrophy, and WMH									
Variable	CDR-SOB n=84			MMSE n=84			ADAS-cog n=84		
	β	*Std. Error*	*P* Value	β	*Std. Error*	*P* Value	β	*Std. Error*	*P* Value
Baseline WMH	0.00011	0.00004	0.02*	-0.0001	0.000074	0.05*	0.0003	0.0002	0.07 ^
Atrophy	-42.61	9.7769	<0.001*	39.71	15.40746	0.01*	-66.54	33.0176	0.05*
Education	-0.1	0.0728	0.1 ^	0.37	0.11487	<0.001*	-0.76	0.2462	0.003*
Gender (Male)	-1.93	0.7306	0.01*	2.05	1.15142	0.08^	-3.98	2.468	0.11 ^
APOE genotype	-0.01	0.0692	0.89	0.027	0.10903	0.8	-0.13	0.2337	0.59
Age of onset	-0.06	0.0543	0.28	0.04	0.08574	0.64	-0.16	0.1838	0.39
Disease duration since first visit	0.004	0.0107	0.7	0.01	0.01689	0.42	-0.02	0.0362	0.5

Table 12: Summary of the mixed-effects models of cognitive change using the predictors baseline WMH volume, atrophy, education, gender, APOE genotype, age of onset, and disease duration as fixed effects. The regression coefficient is shown for the fixed effect followed by the associated P value ("" denotes that the P-value is significant at the 0.05 level, ^ denotes that the P-value is significant at the 0.1 level), and standard errors of coefficients are reported. Regression coefficients represent the number of standard deviations' difference in the cognitive baseline that are associated with a 1-unit change in a predictor measured at baseline. The corresponding protocol of the R output can be found in Table 47-51.*

3.2 Model comparison using Akaike Information Criterion (AIC)

The models which performed better based on the AIC were chosen as the final models. The CDR-SOB model of cognitive decline including WMH, atrophy, gender had an AIC of 1575.67 which was better than the CDR-SOB model of cognitive decline including WMH, atrophy, gender, education, age of onset, apoe, disease duration which had an AIC of 1598.61. The MMSE model of cognitive decline including WMH, atrophy, gender had an AIC of 1966.42 which was better than the MMSE model of cognitive decline including WMH, atrophy, gender, education, age of onset, apoe, disease duration which had an AIC of 1979.68. The ADAS-cog model of cognitive decline including WMH, atrophy, gender had an AIC of 2483.89 which was worse than the ADAS-cog model of cognitive decline including WMH, atrophy, gender, education, age of onset, apoe, disease duration which had an AIC of 2443.39. A post-hoc analysis reveals

weakness in the power of the CDR-SOB model. The smallest detectable effect size (*d*) on the CDR-SOB scale is 0.5 points. The random intercept variance was 3.24, the random slope variance 0.000015, and the residual variance 1.24. The variances were calculated from the corresponding standard deviations (1.79959, 0.00384, 1.114349) which are reported in the linear mixed model output of the CDR-SOB rate of cognitive decline model which included atrophy, WMH, and gender (see appendix). The number of subjects, required for a detectable minimal effect in the CDR-SOB (*d*=0.5) and a desired power of 80%, would be **89**. Thus, the model is slightly underpowered because it contains only **84** subjects. However, based on the comparison of AIC performance, the CDR-SOB model of cognitive decline including WMH, atrophy, gender (AIC=1575.67) performed best of all models (Table 13). Thus, this model was included in further analysis in the next Chapter in which cognitive decline will be associated with mRNA expression and proteins.

Model Comparison							
Model	Df	AIC	Random intercept variance	Random slope variance	Residual variance	Actual model power with n=84, *d*= effect size	Sample size required for a desired power of 80%, p<0.05
CDR-SOB model of cognitive decline including WMH, atrophy, gender	299	1575.67	3.24	0.000015	1.24	0.78, *d*= 0.5	n= 89, *d*= 0.5
CDR-SOB model of cognitive decline including WMH, atrophy, gender, education, age of onset, apoe, disease duration	299	1598.61	3.27	0.000015	1.24	0.78, *d*= 0.5	n= 89, *d*= 0.5
MMSE model of cognitive decline including WMH, atrophy, gender	300	1966.42	8.57	0.000021	3.9	0.87, *d*= 1	n= 69, *d*= 1
MMSE model of cognitive decline including WMH, atrophy, gender, education, age of onset, apoe, disease duration	300	1979.68.	7.84	0.000021	3.89	0.87, *d*= 1	n= 69, *d*= 1
ADAS-cog model of cognitive decline including WMH, atrophy, gender, education, age of onset, apoe, disease duration	299	2443.39	37.96	0.00012	11.56	0.43, *d*= 1	n= 207, *d*= 1
ADAS-cog model of cognitive decline including WMH, atrophy, gender	299	2483.89	41.1	0.00012	11.57	0.43, *d*= 1	n=207, *d*= 1

Table 13: The Model Comparison table summarizes the fit statistics for each model. It is used it to compare several models fitted to the time series. Each row corresponds to a different model. The models are sorted by the AIC statistic.

The random intercept variance, random slope variance, and residual variance were used to calculated the actual power of the models, and the required sample sizes for a desired power of 80%, p<0.05.The effect size (d) is the minimal detectable effect on a scale (here: 0.5 in CDR-SOB points, 1 point in MMSE, and 1 point in ADAS-cog).

The median increase, and Interquartile Range [*IQR*], in points on the CDR-SOB scale, was ~1.33 [*IQR* 2.05] per year[1]. An increase in CDR-SOB points means that there is a decrease in cognitive performance. The median decrease, and *IQR,* in cognitive performance in points on the MMSE scale was 1.19 [IQR 2.47] per year[2]. The median increase, and *IQR,* in cognitive performance in points on the ADAS-cog scale was 1.55 [IQR 5.62] per year[3]. An increase in ADAS-cog points means that there is a decrease in cognitive performance (Table 14).

Median rate of cognitive decline over 1 year in AD patients (AddNeuroMed cohorts)			
Assessment	CDR-SOB n=84	MMSE n=84	ADAS-cog n=84
Median [IQR]	1.33 [2.05]	-1.19 [2.47]	1.55 [5.62]

Table 14: Cognitive decline rates over 1 year measured with three different assessments (CDR-SOB, MMSE, ADAS-cog); median and IQR reported.

1 Calculated from (0.0036 [*IQR*=0.0056] per day * 365.24) ~1.33 [*IQR*=2.05] per year.

2 Calculated from (-0.00328 [*IQR*=0.0067] per day * 365.24) ~ 1.19 [*IQR*=2.47] per year.

3 Calculated from (0.0042 [*IQR*=0.0154] per day * 365.24) ~ 1.55 [*IQR*=5.62] per year.

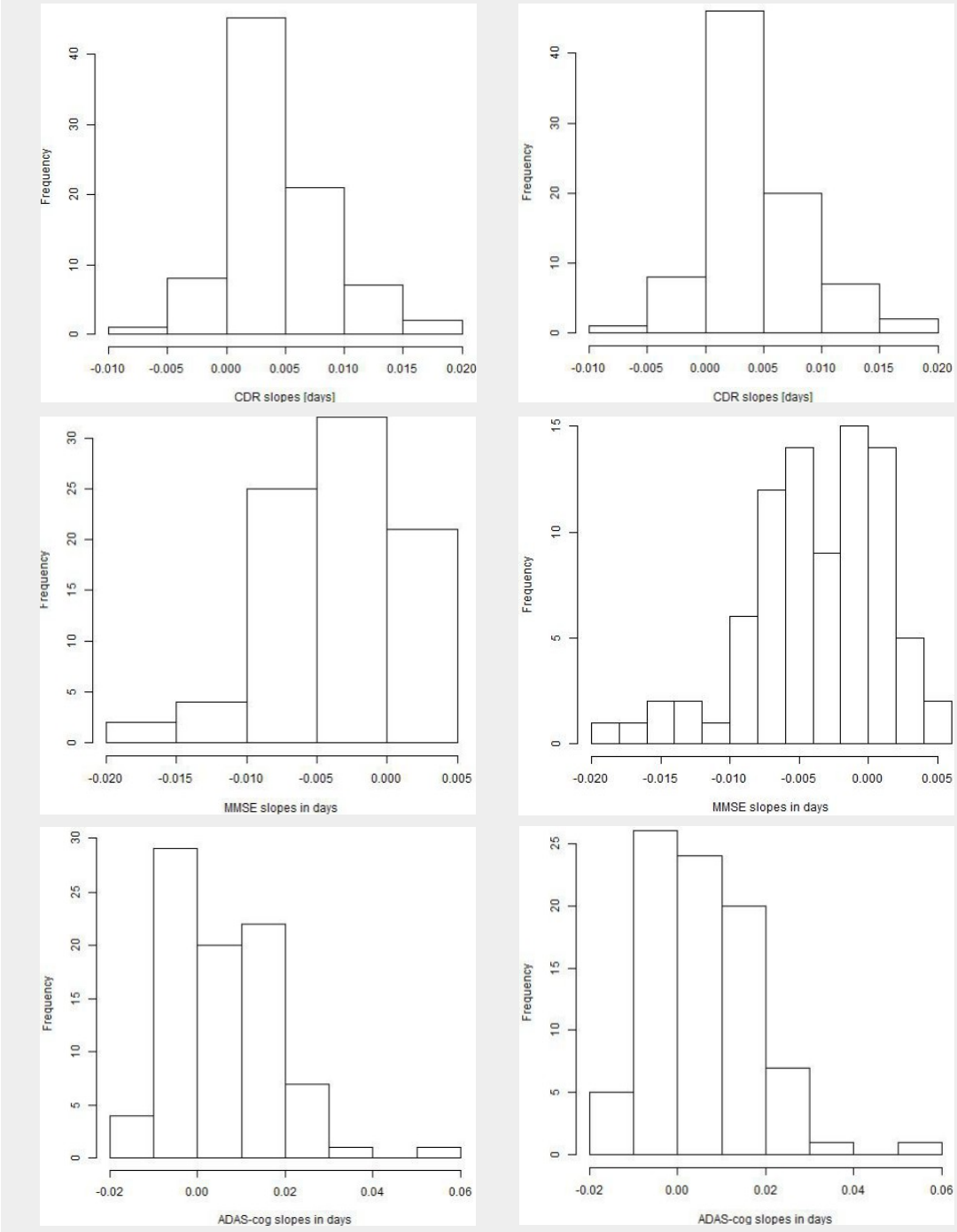

Figure 13: Histogram of cognitive decline slopes including covariates: WMH, atrophy and gender (left column) and including WMH, atrophy, gender, education, age of onset, and disease duration (right column); 1st row: CDR-SOB slopes; 2nd row: MMSE slopes; 3rd row ADAS-cog slopes; plots of the corresponding baseline cognitive decline slopes can be found in the appendix (Figure 19).

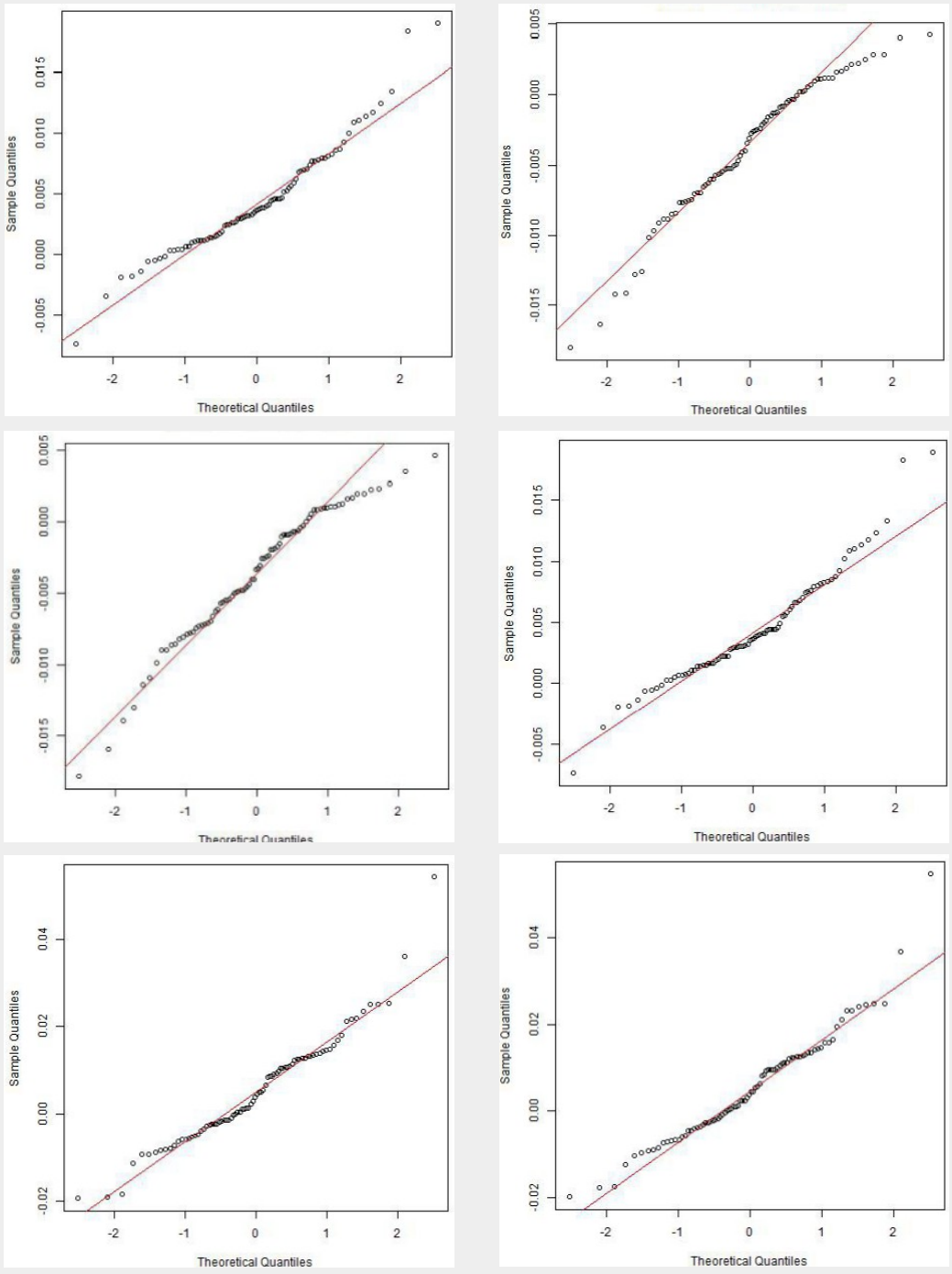

Figure 14: Quantile-Quantile-plot for examining the distribution the of cognitive decline slopes including covariates: WMH, atrophy and gender (left column) and including WMH, atrophy, gender, education, age of onset, and disease duration (right); 1st row: CDR-SOB slopes; 2nd row: MMSE slopes; 3rd row ADAS-cog slopes; the plots of the corresponding baseline cognitive decline slopes can be found in in the appendix (Figure 19).

4 Discussion

The key finding of this study is that WMH volume at baseline was significantly associated with greater subsequent deterioration in global cognition over 1 year, as measured by CDR-SOB, MMSE and ADAS-cog assessments, in a model controlled for atrophy and gender. The finding is significant because it suggests that in a sample with repeated cognitive evaluations and short-term follow-up WMH may be an important predictor of subsequent short-term global cognitive change. Especially, in clinical trials WMH volumes might be an important covariate to consider when selecting patients or evaluating treatment effects.

Of note, there are known limitations of cognitive assessments with regard to measuring short-term follow-up. This can be one possible explanation of why the associations between WMH volumes and cognitive assessment scores are relatively small. It can be speculated that the true effect of WMH-related cognitive decline might be larger than it is possible to show in this small sample containing only 84 subjects.

Another limitation of the model is that its power is slightly under the usually desired minimum power of 80% (Cohen, 1988). Five more subjects would be required to achieve this level of power. If the variable WMH would not be included in the model then a larger number of cognitive decline slopes can be calculated (n= 89) which would meet the required sample size. The WMH variable has only a tiny effect on the rate of cognitive decline (β=0.0001/day) in this particular model. Future studies should focus on subjects with severe WMH volumes. It is more likely to find a large effect on

cognitive decline and vice versa. A recent study claims that subjects with aggressive cognitive decline have more WMH (Tosto et al. 2014).

However, overall the findings agree strongly with prior studies. Especially, greater WMH volumes at baseline were associated with decrease of global cognition in follow-ups (Enzinger et al. 2007) (Fornage et al. 2008) (Silbert et al. 2009) (Carmichael et al. 2010). In the past there has been some controversy regarding the clinical significance of WMH, with some clinicians even considering a high amount of WMH normal in old age (Erkinjuntti et al. 1994). In the recent Carmichael study, baseline WMH were significantly correlated with greater subsequent cognitive decline over 1 year measured by MMSE (β= -0.096; P<0.001) and ADAS-cog (β=-0.034; P<0.05). Also in this study the predictor values were relatively small indicating a small effect.

A study by Brickman *et al.* has shown that the degree of baseline WMH (β= -0.173; P=0.03), atrophy (β= -0.316; P=0.04), and the interaction with atrophy predicted the rate of cognitive decline measured by MMSE scores over 6 follow-up evaluations with 1-year intervals. The MMSE scores decreased by an average of 3.5 points per year.

In the current study, there was an average decrease of ~1.4 MMSE points per year in the subjects of the AddNeuroMed cohort indicating that the general decline was less than half as accelerated as in the cohort from the Massachusetts General Hospital (Erkinjuntti et al. 1994). Atrophy was clearly the most influential variable in the cognitive decline models calculated in the current study, as indicated by the large weight of the predictor

atrophy, with a magnitude from of 30 in the CDR-SOB and 39 MMSE model and significant p-values (P<0.05). In comparison to Brickman's model, here, interactions between WMH and atrophy were not significant.

WMH appear to be very prevalent in aged persons, including AD patients (Capizzano et al. 2004; Van der Flier et al. 2005; Yoshita et al. 2006; Matsusue et al. 2006; Gouw et al. 2008; Holland et al. 2008; Gold et al. 2012). Also in the current study there was a positive correlation between WMH volumes and age, R^2=0.29, n=100, P<0.05.

Higher education can influence the rate of cognitive decline, primarily affecting executive speed and memory (Scarmeas et al. 2006). Carmichael and colleagues found that education was significant as a covariate in their CDR-SOB (β= -0.013; P= 0.01), but not in their ADAS-cog or MMSE models (Carmichael et al. 2010). In the current study, the significant of effect of education is suggested in the MMSE (β= 0.349; P<0.05) and ADAS-cog model (β= -0.075; P<0.05).

The prevailing hypothesis is that WMH represent small ischemic cerebrovascular disease and vascular changes affecting perfusion and blood flow (Provenzano et al. 2013).

White matter contains 4-times more vascular Aβ which might cause micro-vascular damage. It is possible that vascular Aβ increases the risk for the development of AD and reflects the presence of WMH and the vascular problems associated with WMH (Roher

et al. 2002). Also other studies confirm that high WMH burden and vascular problems convey increased risk for the development of AD (De Leeuw et al. 2005; Gold et al. 2012; Weinstein et al. 2013).

In the current study, patients had a mild cardiovascular profile because subjects with stroke or severe vascular pathology were excluded from the study to analyse an overall homogeneous AD group without the influence of comorbidities.

Baseline WMH burden is a potentially important covariate in predicting an AD patient's rate of disease progression. However, WMH progression is possibly another influential variate. Several studies have shown that white matter changes progress over time (Fazekas & Wardlaw 2013) (Silbert et al. 2009). Carmichael *et al.* found significant associations between progression of WMH and cognitive disease progression (β= -0.1; P<0.001). In the current study, only baseline WMH volumes were available but future work can include the continuation of this work with longitudinal WMH measures.

Structural and functional imaging have the capabilities to detect AD related pathological changes in brain regions which are particularly affected in AD (Scheltens & Korf 2000) (Scheltens & Korf 2000) (Gootjes et al. 2004) (Henneman et al., 2009) (Prins et al. 2004) (Zhang et al. 2009). Earlier studies did not find evidence of AD-specific distribution of WMH (Henneman et al. 2009) whereas recent studies suggest that the extent and distribution of WMH in frontal and temporal brain regions is related to AD severity and diminished cognition (Kim et al. 2011). WMH in AD have been found to be

distributed in periventricular brain regions (DeCarli et al. 2005; Holland et al. 2008), in regions adjacent to the posterior corpus callosum (Scheltens et al. 2000; Ferrarini et al. 2008; Zhang et al. 2009; Silbert et al. 2009), in parahippocampal white matter and in caudal portions of the cingulum (Gold et al. 2012). Brain regions with increased white matter dis-integrity correlate with measures of decreased cerebral blood flow/ metabolism in these areas (Chao et al. 2013) (Provenzano et al. 2013) (Kim et al. 2011).

More insights into the pathophysiology of WMH and their exact impact on cognitive decline are clearly needed to reduce the burden of disability associated with this common condition in neurodegenerative diseases.

Although evidence from neuro-imaging studies using temporal models for short term prediction of individual disease decline is available larger studies are required. One difficulty is that cognitive changes often occur in a non-linear, step-wise manner and are highly variable. Jack and colleagues propose a model for the temporal order in which changes relating to the disease occur (Jack et al. 2013). Disease progression models can contribute to understanding these changes over time and how and when the different markers correlate with each other. Such models provide the groundwork for further evaluation of study design and analysis methodologies for clinical trials in AD (and MCI) (Ito et al. 2010).

WMH and AD share many of the same risk factors, both are hopefully preventable if intervention takes place early enough. Initiatives guiding the future direction of research

into Alzheimer's disease envisage a panel of preventative strategies such as recognising vascular, lifestyle, psychological, and genetic risk factors of aged individuals (Ito et al. 2010). Some of these risks can be possibly targeted with new drug treatments, psychological help, and lifestyle advice.

In the current work, global WMH have been correlated with measures of cognitive change, and in addition, in the next Chapter, with mRNAs and proteins circulating in blood. In future, cerebral structural and functional imaging combined with blood-based markers could contribute to provide individual predictions for disease progression.

Chapter 2. Identification of a mRNA and protein panel associated with WMH and AD-cognitive decline

1 Introduction

1.1 Transcriptomic markers

Transcriptomic markers are based on measurements of RiboNucleic Acid (RNA) obtained from blood samples. An RNA biomarker indicates the level of expression of a gene, i.e., the amount of messenger RNA (mRNA) produced. RNA, together with DNA, constitute the building blocks of life as we know it, and play various essential roles in the coding, decoding, regulation, and expression of genes. We often refer to these RNA molecules present in a cell at *one point in time* as the transcriptome, varying by cell type, tissue, organ, environment, treatment, time of day and age. The fact that we can identify the work of cell at a particular time point makes RNA interesting as a biomarker.

Advantages of the transcriptome (all RNA expressed in a cell) are that current techniques allow multiplexing more RNA at once than proteome-related techniques. However, in this study, only mRNA is measured, not the other RNA types which are present in a cell. Usually, only transcripts which are statistically significantly different, under two or more conditions are taken into account for further analysis.

The limitation of these transcripts are (a) many genes are generally turned off in a

certain cell-type or tissue as a result of cellular differentiation; (b) some life sustaining genes are always turned on and may produce confusing transcript/proteins signals; it is necessary to discount these signals in the analysis; (c) cells can control protein production in many ways, not only via altering the amount of mRNA, thus the expression level might not correspond to the protein concentration.

Previous studies identified a number of transcriptomic markers associated with AD: these molecules were implicated in pathways such as immunity, long-term potentiation, mitrochondrial function, phosphrylation, RAS-MAPK-ERK, and the ubiquitin protease system (Altar et al. 2009; Grünblatt et al. 2009; Chen et al. 20011; Lunnon et al. 2013; Kam et al. 2013). For instance, RAS-MAPK-ERK pathway is associated with the development of many cancers by sending off/on signals to proteins (Imtiaz et al. 2014).

Mitochondria, the energy production facility of the cell, must function properly for maintaining many bodily processes. Molecular signatures indicating their dysfunction can be seen in blood of AD patients (Lunnon et al. 2013).

The ubiquitin protease system is required for regulating immune responses. Dysfunction of ubiquitination has been observed in AD (Upadhya & Hegde 2007).

Memory is severely affected in AD, thus it is plausible that markers can be found in blood which influence long-term potentiation (the underlying synaptic process of memory formation). It is also possible that post-translational modification play a role in AD (Grünblatt et al. 2009). Below an overview of transcriptomic markers, their

association with AD, and pathways they are implicated in (Table 15).

Biomarker	Linked with AD	Pathways	References
Neurotrophin receptors fibroblast growth factor glutamate receptors dopamine receptors	Gene and pathway targets replicated in expression profiling of human postmortem brain, animal models, and cell culture studies	Ubiquitin-proteosome system, protein kinase C and RAS pathways	(Dhillon et al. 2007)
CB2 (Cannabinoid-receptor 2) H3-histone	Increased in subjects with lower MMSE	MAPK-ERK, long-term potentiation Post-translational modifications	(Altar et al. 2009)
ABCB1	Positively correlated with MMSE	Immunity	(Grünblatt et al. 2009)
48 genes	Classification AD *versus* controls	Mitchondrial, oxidative phosporylation, and translation	(Chen et al. 2011)
FcyRIIb	Mediates amyloid-β and memory impairment	Long-term potentiation	(Lunnon et al. 2013)

Table 15: Summary of transcriptomic markers for Alzheimer's disease from literature

1.2 Proteomic markers

Proteomics has contributed enormously to a deeper understanding of the pathological processes in AD and is playing an important role in identifying biomarkers for early diagnosis, monitoring progression, and response to therapy (Li & Wong 2001). Proteomic markers are based on measurements of proteins, usually obtained from blood. A protein biomarker indicates the amount of translated protein of each gene in a cell, tissue, or organism. Proteins play vital roles in diseases. Certain protein levels in a cell can be indicators of the presence of a disease. An advantage of proteins found in blood plasma is that changes in blood can reflect changes in the brain to a certain degree because the brain is not entirely segregated from the periphery; recent evidence suggests there is vivid communication between brain and peripheral blood (Lovestone et al. 2007) (Brunden et al. 2011) (Cortes-Canteli et al. 2012) (Banks 2012) (Wu et al. 2013).

1.2.1 Blood-based protein markers

Ray *et al.* have measured eighteen signalling proteins that were found to be different in early stages of AD: systemic dysregulation, hematopoiesis, immune responses, apoptosis, and neuronal support in pre-symptomatic Alzheimer's disease (Ray et al. 2007). These proteins have been used for identification of patients with AD, or for predicting the onset of AD in presymptomatic patients, with up to 90% accuracy. Also patients with mild cognitive impairment were analysed and it has been found that progression to AD could be predicted with 81% accuracy in patients even 6 years before their clinical diagnosis.

However, in a reproduction study Björkqvist and colleagues quantified the same proteins that have been already analysed by Ray et al. 2007 but did not find evidence of them being useful as a good biomarker: Only three proteins (EGF, PDG-BB and MIP-1δ) were different in plasma between controls and AD (Björkqvist et al. 2012).

Also Soares *et al.* tested the reproducibility of the original 18-analyte panel from Ray et al. using a bead-based multiplex technology. Their results suggest diagnostic accuracy using the subset was 61%. Multivariate analysis of an 89-analyte multivariate panel yielded a diagnostic accuracy of 70% suggesting a plasma-based AD signature that may be a useful screening tool in future but it still too weak for AD diagnosis based on this set (Soares et al. 2009).

O'Bryant *et al.* developed an algorithm for detecting the presence of AD which performed with 80% sensitivity and 91% specificity based on a panel of 30 proteins (O'Bryant et al. 2010; O'Bryant et al. 2011). The panel included many proteins which

are usually implicated in inflammation and vascular events. In addition, these proteins correlated with neuropsychological scores.

Laske *et al.* applied a multivariate approach (based on a support vector machine algorithm) to identify three most discriminative parameters (cortisol, von Willebrand factor, oxidized low-density lipoprotein (LDL) antibodies). They could classify between AD and cognitively normal subjects with an accuracy of 81.7%/ 87.1% in the training/independent test dataset. Later they found exaberrant levels of the soluble TNF receptor 1 (sTNF-R1) in blood of AD patients *versus* controls (Laske et al. 2011) (Laske 2013).

Doecke *et al.* found significantly altered concentrations of 18 proteins in AD subjects compared to healthy controls (Doecke et al. 2012). The plasma samples were drawn from 207 AD patients and 754 controls as part of Australian Imaging, Biomarker & Lifestyle study (AIBL). A complete independent validation cohort was used: the Alzheimer's disease neuroimaging initiative (ADNI) in which they achieved 80% accuracy, in 108 AD patients and 57 controls. However, in the ADNI cohort age, gender and APOE ε4 genotype alone could predict the diagnosis of Alzheimer's disease with an accuracy of 77%. Thus, these proteins discovered could explain only 3% of the differences found in blood.

Currently, quantitative mass-spectrometry assays are under development and in future the investigation of post-translational modifications of proteins will be feasible (Snyder et al. 2014).

1.2.2 Protein markers from brain tissue

Research on brains from AD individuals has identified not only the two hallmark molecules, amyloid deposits and tau pathology, but also changes in astrocytes, microglia, immune responses implicating cytokines and chemokines, and reactive oxigen species (Snyder et al. 2014) (Heneka et al. 2010). In AD patients and other individuals with brain injury excessive load of pro-inflammatory cytokines such as TNF-α have been measured accelerating the rate of cognitive disease progression (Holmes 2009).

Below an overview of blood and brain protein markers (Table 16).

Biomarker	Link with AD	Pathways	References	
α-1-antitrypsin	Enhances the formation of amyloid-fibrils	Inflammation	(Musunuri et al. 2014)	(Thambisetty et al. 2010)
α-2-macroglobulin		Inhibitor of coagulation	(Hye et al. 2006)	
Apolipoprotein E		Cholesterol metabolism		
Complement C3		Innate immunity		
These four proteins above ...	... replicate in multiple studies		(Kiddle et al. 2014)	
Serpin F1 (pigment epithelium-derived factor) Complement C1 inhibitor	Down-regulated in AD blood		(Maes et al. 2006)	
18 proteins	Different in AD *versus* controls	Systemic deregulation, hematopoiesis, immune responses, apoptosis, and neuronal support	(Cutler et al. 2008)	
Clusterin	Amyloid chaperone protein	Immune response	(Ray et al. 2007)	
Homocysteine	Increased in blood from female AD patients		(Obulesu & Jhansilakshmi 2014)	
Red cell folate	decreased in AD blood			
Fibrinogen	Vascular dysfunction Linked with brain atrophy Altered hemostasis in AD	Coagulation Binding of Aβ with fibrinogen leads to increased fibrinogen	(Wood 2010) (Thambisetty et al. 2011) (Faux et al. 2011)	

		aggregation, Aβ fibrillization, and formation of degradation-resistant fibrin clots	
β2-microglobulin		Interferon-gamma-mediated signalling pathway	(Cortes-Canteli et al. 2012)
Cystatin C	Inhibits Aβ oligomerization and amyloidogenesis, upregulated in response to injury	Apoptosis	(Zellner et al. 2009)
Brain-derived neurotrophic factor 1	Role in neurodegeneration	TrkB pathway	(Kaur & Levy 2012)
Septin-3, septin-2, septin-5, dihydropteridine reductase, and clathrin heavy chain 1	Altered levels in AD brain	Energy metabolism, glycolysis, oxidative stress, apoptosis, signal transduction, and synaptic functioning	(Honea et al. 2013)

Table 16: Summary of candidate protein markers for Alzheimer's disease from literature

1.3 Aims

An ideal blood marker for AD should be easily obtainable and non-invasive. Therefore, there is a clear need to search for biomarkers in blood. Blood markers for AD offer very promising perspectives, however, do not yet provide sufficiently validated prognostic information. In this work, the aims are to provide some transcriptomic and proteomic candidates, reflecting disease progression in AD, for further investigation in research and maybe even in a clinical trial.

One AD-related (but not AD-specific) condition is WMH burden which could impact the prediction for the rate of cognitive decline. The study aims to find a panel of mRNA and proteins associated with WMH. The current study could reveal important clues to the course of AD pathology. Our aims are formulated accordingly:

(1) Applying statistical models for the identification of mRNA transcripts and proteins from blood correlated with WMH, firstly in a mixed group of AD, MCI, and controls,

regardless of disease status, and secondly in the subgroup of AD.

(2) Applying statistical models for the identification of mRNA transcripts and proteins correlated with AD disease decline using the cognitive decline slopes previously generated.

2 Methods

2.1 Cohorts & subjects

The detailed inclusion/exclusion criteria for AD have been described in the previous chapter and by Simmons and colleagues (Simmons et al. 2011).

The inclusion criteria for MCI and control subjects were (Simmons et al. 2011): (1) MMSE score range between 24 and 30; (2) geriatric depression scale score less than or equal to 4/5; (3) subject aged 65 years or above; (4) subjects' medication; and (5) good general health.

The exclusion criteria for MCI and control subjects were (Simmons et al. 2011): (1) subjects meet the DSM-IV criteria for dementia; (2) significant neurological or psychiatric illness, and (3) significant systematic illness or organ failure. The distinction between MCI and controls was based on two criteria: (1) subject scores 0 on the CDR scale = control and (2) subject scores 0.5 on CDR scale = MCI. Control and MCI subjects were further assessed using the CERAD (Consortium to Establish a Registry for Alzheimer's Disease) battery (Fillenbaum et al. 2008).

Different versions of the cognitive assessments were used to avoid practice effects. If the same cognitive tests would have been used 5 times a year it would have been likely that the patients can remember some of the questions.

2.2 Workflows and distribution characteristics

2.2.1 WMH versus mRNA transcripts in AD, MCI, and controls

74 AD, 65 MCI, and 45 normal controls, matched on WMH volumes and mRNA measures, from the AddNeuromed study, were included (Table 17).

<table>
<tr><td></td><td colspan="2">Workflow WMH versus mRNA transcripts in AD, MCI, controls</td><td></td></tr>
<tr><td colspan="2">Subjects with WMH measures (AddNeuroMed)
Total AD MCI CTL
245 100 87 58</td><td colspan="2">Subjects with mRNA measures (AddNeuroMed)
Total AD MCI CTL
331 106 117 108</td></tr>
<tr><td></td><td colspan="2">1. Matching subjects</td><td></td></tr>
<tr><td colspan="4">Total AD MCI CTL
184 74 65 45</td></tr>
<tr><td></td><td colspan="2">2. Randomisation of subjects [random.org/lists]</td><td></td></tr>
<tr><td></td><td colspan="2">3. Dividing in training and validation set</td><td></td></tr>
<tr><td colspan="2">Training set
138 subjects, 6282 mRNA transcripts</td><td colspan="2">Validation set
46 subjects, 6282 mRNA transcripts</td></tr>
<tr><td></td><td>4. Feature selection on training set with WEKA automatic feature selection classifier Cfs subset eval, BestFirst, Crossval 25-fold, seed 1</td><td></td><td></td></tr>
<tr><td colspan="2">Training set with selected features
138 subjects, 18 mRNA transcripts</td><td colspan="2">Independent validation set
46 subjects, 18 mRNA transcripts</td></tr>
<tr><td></td><td>5. Nesting with WEKA CVParameterSelection:numFold =10, seed=1; Linear Regression, M5 enabled</td><td></td><td></td></tr>
<tr><td></td><td>6.a Evaluation on training set</td><td>6.b Re-evaluation on validation set</td><td></td></tr>
<tr><td colspan="2">R^2, Root mean squared error of evaluation on training set</td><td colspan="2">R^2, Root mean squared error of evaluation on validation se</td></tr>
</table>

Table 17: Workflow WMH volume as dependent variable versus mRNA transcripts in AD, MCI, and controls

There is only a marginally significant difference in WMH volumes by disease status (Figure 15).

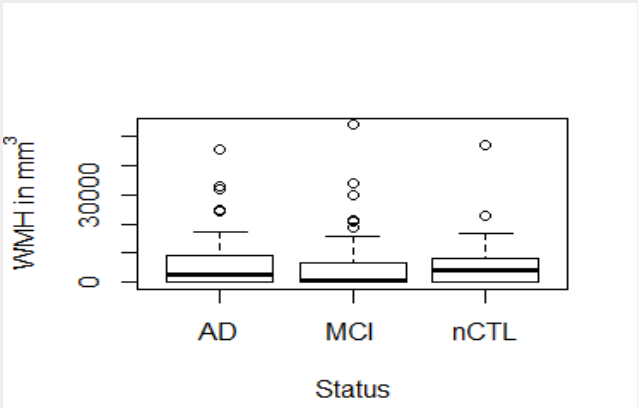

Figure 15: Boxplot of WMH volume by disease status used in the analysis WMH versus mRNA, χ2 (2, N = 184) = 5.77, P = 0.05.

2.2.2 WMH versus mRNA transcripts in AD

74 AD, matched on WMH volumes and mRNA measures from the AddNeuromed study, were included (Table 18). Of note, the training group and validation group are a subset of the mixed group (section 2.2.1).

	Workflow WMH *versus* mRNA transcripts in AD	
Subjects with WMH measures (AddNeuroMed) Total AD 245 100	Subjects with mRNA measures (AddNeuroMed) Total AD 331 106	
	1. Matching subjects	
Total AD 184 74		
	2. Randomisation of subjects [random.org/lists]	
	3. Dividing in training and validation set	
Training set	Validation set	

	Workflow WMH *versus* mRNA transcripts in AD		
53 subjects, 6282 mRNA transcripts		21 subjects, 6282 mRNA transcripts	
	4. Features were used from the previously analysed mixed AD, MCI, controls are used because the AD group is a subset of the training of the AD|MCI|CTL group.		
Training set with selected features 53 subjects, 18 mRNA transcripts		Independent validation set 21 subjects, 18 mRNA transcripts	
	5. Nesting with WEKA CVParameterSelection:numFold =10, seed=1; Linear Regression, M5 enabled		
	6.a Evaluation on training set	**6.b Re-evaluation on validation set**	
R^2, Root mean squared error of evaluation on training set		R^2, Root mean squared error of evaluation on validation set	

Table 18: *Workflow WMH volume as dependent variable versus mRNA transcripts in AD*

2.2.3 WMH versus proteins in AD, MCI, and controls

89 AD, 54 MCI, and 39 normal controls, matched on WMH volumes and protein measures, from the AddNeuromed study and the Dementia Case Register were included (Table 19).

	Workflow WMH *versus* proteins in AD, MCI, controls		
Subjects with WMH measures (AddNeuroMed and Dementia Case Register) Total AD MCI CTL 353 142 100 111		Subjects with protein measures (AddNeuroMed and Dementia Case Register) Total AD MCI CTL 375 196 69 110	
	1. Matching subjects		
Total AD MCI CTL 182 89 54 39			
	2. Randomisation of subjects [random.org/lists]		
	3. Dividing in training and validation set		
Training set 136 subjects, 1016 proteins		Validation set 46 subjects, 1016 proteins	
	4. Feature selection on training set with WEKA automatic feature selection classifier Cfs subset eval, BestFirst, Crossval 25-fold,		

	Workflow WMH *versus* proteins in AD, MCI, controls		
	seed 1		
Training set with selected features 136 subjects, 27 proteins		Independent validation set with selected features 46 subjects, 27 proteins	
	5. Nesting with WEKA CVParameterSelection:numFold =10, seed=1; Linear Regression, M5 enabled		
	6.a Evaluation on training set	**6.b Re-evaluation on validation set**	
$R^{2,}$, Root mean squared error of evaluation on training set		$R^{2,}$, Root mean squared error of evaluation on validation set	

Table 19: Workflow for WMH volume as dependent variable versus proteins in a mixed group of AD, MCI, and controls

There is only a marginally significant difference in WMH volumes by disease status (Figure 16).

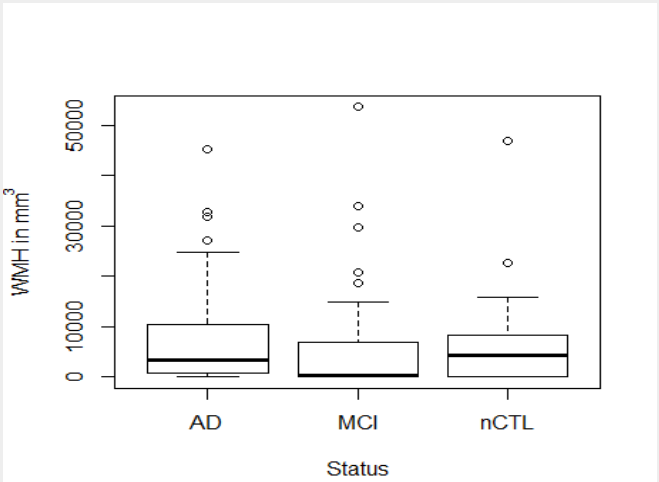

Figure 16: Boxplot of WMH volume by disease status used in workflow WMH versus proteins, χ2 (1,N = 182) = 6.22, P = 0.04.

2.2.4 WMH versus proteins in AD

89 individuals with AD, matched on WMH volumes and protein concentrations from the AddNeuroMed study and the Dementia Case Register, were included (Table 20). Of note, the training group and validation group are a subset of the previous mixed group in

order to test the previously identified proteins (section 2.2.3).

	Workflow WMH *versus* proteins in AD subjects		
Subjects with WMH measures (AddNeuroMed and Dementia Case Register) Total AD 353 142		Subjects with protein measures (AddNeuroMed and Dementia Case Register) Total AD 375 196	
	1. Matching subjects		
Total AD 182 89			
	2. Randomisation of subjects [random.org/lists]		
	3. Dividing in training and validation set		
Training set 59 subjects, 1016 proteins		Validation set 30 subjects, 1016 proteins	
	4. Features were used from the previously analysed mixed AD, MCI, controls are used because the AD group is a subset of the training of the AD|MCI|CTL group.		
Training set with selected features 59 subjects, 27 proteins		Independent validation set with selected features 30 subjects, 27 proteins	
	5. Nesting with WEKA CVParameterSelection:numFold =10, seed=1; Linear Regression, M5 enabled		
	6.a Evaluation on training set	**6.b Re-evaluation on validation set**	
R^2, Root mean squared error of evaluation on training set		R^2, Root mean squared error of evaluation on validation set	

Table 20: *Workflow for WMH volume as dependent variable versus proteins in AD subjects*

2.2.5 Rate of cognitive decline versus mRNA in AD

62 individuals with AD, matched on the previously calculated CDR-SOB rates of cognitive decline and mRNA expression from the AddNeuromed study, were included (Table 21).

	Workflow rate of cognitive decline versus mRNA transcripts		
AD subjects with WMH measures (AddNeuroMed) 100		AD subjects with mRNA measures (AddNeuroMed) 331	
	1. Matching subjects		
62 AD			
	2. Randomisation of subjects [random.org/lists]		
	3. Dividing in training/validation		
Training 46 subjects, 6282 mRNAs		Validation 16 subjects, 6282 mRNAs	
	4. Feature selection on training set WEKA feature selection classifier Cfs subset eval, BestFirst, Crossval 25-fold, seed 1		
Training set with selected features 46 subjects, 27 mRNAs		Independent validation set 16 subjects, 27 mRNAs	
	5. Nesting with WEKA CVParameterSelection:numFold=10, seed=1; Linear Regression, M5 enabled		
	6.a Evaluation on training set	**6.b Re-evaluation on validation set**	
R^2, Root mean squared error of evaluation on training set		R^2, Root mean squared error of evaluation on validation set	

Table 21: Workflow for rate-of-cognitive decline as dependent variable versus mRNA transcripts

2.2.6 Rate of cognitive decline versus proteins in AD

63 individuals with AD, matched on the CDR-SOB rates of cognitive decline and protein measures from the AddNeuromed study, were included (Table 22).

	Workflow rate of cognitive decline versus proteins		
AD subjects with cognitive decline measures (AddNeuroMed) 100		AD subjects with protein measures (AddNeuroMed) 282	
	1. Matching subjects		
63 AD			

	Workflow rate of cognitive decline versus proteins		
	2. Randomisation of subjects [random.org/lists]		
	3. Dividing in training/validation		
Training 47 subjects, 1016 proteins		Validation 16 subjects, 1016 proteins	
	4. Feature selection on training set WEKA feature selection classifier Cfs subset eval, BestFirst, Crossval 25-fold, seed 1		
Training set with selected features 47 subjects, 27 proteins		Independent validation set 16 subjects, 27 proteins	
	5. Nesting with WEKA CVParameterSelection:numFold=10, seed=1; Linear Regression, M5 enabled		
	6.a Evaluation on training set	**6.b Re-evaluation on validation set**	
R^2, Root mean squared error of evaluation on training set		R^2, Root mean squared error of evaluation on validation set	

Table 22: Workflow for rate-of-cognitive decline as dependent variable versus proteins

2.3 Illumina Microarray Analysis

Lunnon *et al.* have previously performed the microarray analysis (Lunnon et al. 2013). This was not in the scope of this PhD thesis. The microarray data were extracted using Human HT-12 v3 Expression BeadChips (Illumina). The beadchips consisted of 48,803 probes and were optimised to process data from RefSeq (Build 36.2, Rel 22) and the UniGene (Build 199) databases. The TotalPrep RNA Amplification Kit (Ambion) was used to synthesize cDNA from 200 ng total RNA followed by amplification and biotinylation of cRNA and hybridization. Following hybridization, quality control of the bead arrays was carried out using the R Bioconductor package "ComBat". The gene

expression values were variance-stabilization transformed and quantile normalized using the R Bioconductor package lumi (Du et al. 2008). The data were adjusted for RNA integrity number, RNA concentration, gender and age. Only probes showing an expression signal across all samples were kept which left 6,282 probes. A total of 30 chips were excluded from further analysis for reasons including: very low BeadChip detection rate, disparity in XIST gene expression gender, inconsistencies in subjects characteristics (Lunnon et al. 2013).

2.4 SOMAscan protein measures

The protein measurements were previously generated and analysed by Sattlecker and colleagues (Sattlecker at al. 2013). The work describe in this section was not in the scope of this thesis. Protein levels in plasma were measured using a Slow Off-Rate Modified Aptamer (SOMAmer)-based multiplexed proteomic assay (SomaLogic, Inc, Boulder, Colorado). For measuring plasma proteins this array (SOMAscan) uses chemically modified nucleotides: a signature of protein concentration is transformed into a DNA aptamer concentration, a nucleotide signal (relative fluorescence units) quantifiable on the DNA microarray using relative florescence. This assay uses unique nucleotide sequences recognizable by specific hybridization probes (Kiddle et al. 2014). This assay is capable of simultaneously reading out several thousand proteins even in small sample volumes (15 μL serum or plasma). The current assay is capable of measuring 813 proteins with low limits of detection (1 pM median), 7 logs of overall dynamic range (~100 fM–1 μM), and 5% median coefficient of variation. The median

lower and upper limits of quantification were ~1 pM and ~1.5 nM in buffer, and ~2.95 pM and ~1.5 nM for a subset of the somamers in plasma, full details are given in Gold *et al*. (Gold et al. 2010). Quality control was performed at the sample and SOMAmer level, and involved the use of control SOMAmers on the microarray and calibration samples. At the sample level, hybridization controls on the microarray were used to monitor sample-by-sample variability in hybridization, while the median signal over all SOMAmers was used to monitor overall technical variability. The resulting hybridization scale factor and median scale factor were used to normalize data across samples. The acceptance criteria for these values were 0.4-2.5, based on historical trends in these values. SOMAmer-by-SOMAmer calibration occurs through the repeated measurement of calibration samples, these samples were of the same matrix as the study samples, and were used to monitor repeatability and batch to batch variability. Historical values for these calibrator samples for each SOMAmer were used to generate a calibration scale factor. The acceptance criteria for calibrator scale factors is that 95% of SOMAmers had a calibration scale factor within ±0.4 of the median. The assay required 8μL of plasma from each sample. A single assay was used per plasma sample, and thus no technical replicates were performed. Additionally, the samples were run in two batches ensuring an even mix of diagnosis groups in each batch. The protein measures were normalised. Principal component analysis showed that protein measures were affected by study centre and thus either adjusted for centre using linear regression or added centre as a covariate in all downstream analysis (Sattlecker at al. 2013). This method has enabled to measurements of the concentration level of 1,016 human proteins representing different molecular pathways and gene families.

2.5 MRI

Detailed information regarding data acquisition, pre-processing, and quality control assessment, and WMH extraction have been described in the previous Chapter (pages 82 & 84).

2.6 Multivariate analysis with WEKA software

WEKA software was used for performing feature selection and linear regression (Holmes et al. 1994) (Hall et al. 2009) (Witten et al. 2011).

2.6.1 Feature selection search method

A total of 6,282 mRNA transcripts and 1,016 proteins were included in the feature selection which was performed, in the training set, with BestFirst Search method and CFS subset evaluator, with the options 10-fold crossvalidation and seed 1, resulting in a list of mRNAs and proteins sorted by the number of folds they appeared in; if they appeared at least 7 out of 10 times they were included in the analysis. The Best first attribute selection method searches the space of attribute subsets by greedy hillclimbing augmented with a backtracking facility. Setting the number of consecutive non-improving nodes allowed controls the level of backtracking done. Best first may start with the empty set of attributes and search forward, or start with the full set of attributes and search backward, or start at any point and search in both directions (by considering all possible single attribute additions and deletions at a given point). Here we use forward as direction. The threshold for keeping mRNAs and proteins in the analysis was if they were found in at least 8 of 10 folds.

CFS Subset Evaluator, the standard attribute selection method in WEKA, evaluated the worth of a subset of attributes by considering the individual predictive ability of each feature (mRNA, protein) along with the degree of redundancy between them. Subsets of features that are highly correlated with the dependent variable (here: WMH or CDR-SOB) while having low intercorrelation are preferred (Witten et al. 2011). The standard options were used: locallyPredictive=TRUE (identifies locally predictive attributes and iteratively adds attributes with the highest correlation with the dependent variable as long as there is not already an attribute in the subset that has a higher correlation with the attribute in question) and missingSeparate =False (counts for missing values are distributed across other values in proportion to their frequency).

2.6.2 Linear regression, M5 algorithm, and parameter tuning

A number of options specific to the WEKA classifier function "Linear Regression" were tested and the most suitable applied using a meta learner Cross-ValidatedParameterSelection (CVPS) which gives an unbiased performance estimate for every step involved in fitting the model while optimising the parameters. The options for parameter settings included:

-S sets the attribute selection method to use: (0) the default method is M5' (builds trees whose leaves are associated to multivariate linear models and the nodes of the tree are chosen over the attribute that maximizes the expected error reduction, given by the Akaike information criterion (AIC) – a measure of the relative goodness of fit of a

statistical model (Akaike, 1973); (1) no selection; (2) greedy, a heuristic method which finds a solution after a number of steps.

-C (0) co-linear attributes are removed; (1) co-linear attributes are not removed.

-R sets the ridge parameter (default = 1.0e-8). The goal is to circumvent the problem of predictors' co-linearity. The following parameters were tested and applied using CVPS (Table 23) (Figure 17).

Parameters	***Test parameters***	***Optimized parameters (applied in the analysis)***
Ridge	R -5.99 5.0E-4 5.0	R 4.99
Co-linearity	C 0.0 1.0 1.0	C 0.0
Attribute **S**election method	S 0.0 1.0 2.0	S 0.0

Table 23: Regression and parameter tuning: test- and optimized parameters

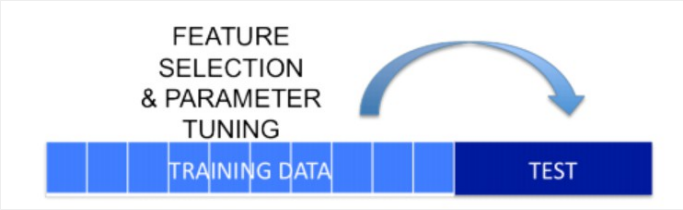

Figure 17: Parameter tuning using a 10-fold cross-validation. Using meta learners such as CVParameterSelection, a nested cross-validation is obtained in which parameters and features are selected using an inner run of cross-validation on each training fold of the outer evaluation cross-validation.

2.6.3 Model output and reporting of results

The model output consisted of: (1) run information: a list of information giving the learning scheme options, relation name, instances, attributes and test mode that were

involved in the process; (2) model: a textual representation of the classification model that was produced on the training data; (3) summary: a list of statistics (correlation coefficient and errors), summarizing how accurately the model was able to predict the true target of the instances under the chosen test mode.

Reporting of results included correlation coefficient, mean absolute error, root mean squared error, relative absolute error in %, root relative squared error in %, number of subjects, number of features, type of target (WMH or cognitive decline slope) were reported for the training, 10-fold cross-validated training, and an independent validation set. The final model (based on the training data), identified with parameter tuning and regression, was exported to R to extract the test statistics. This was not possible for the cross-validated model and validation model because they are directly built from the training model and it was not foreseen to export these models from WEKA.

Power and sample size analysis was performed using G*Power 3.1.7 (Faul et al. 2007 & 2009). A protocol of power analysis was reported with estimated sample sizes for the validationsets.

Observations for WEKA

Having a training set and a validation set is regarded as the gold standard. In the current study, after generating and analysing training and validation sets, it be became apparent that the sample sizes for the validation sets were too low (based on power analysis) which is a limitation of the performed analyses (Table 24). Future work should include the collection of more data for larger validation sets. Another possibility would have

been to use only larger training sets until further data is collected and apply a cross-validation. Cross-validation can give an insight how data are expected to generalize in an independent validation set but they are no replacement for validation in an independent dataset. Cross-validation is a method to predict model fit for a hypothetical validation set when an explicit set is not available.

Study	*Actual size of validation set*	*Ideal size of validation set*
WMH/mRNA (AD, MCI, controls)	46	52
WMH/mRNA (AD)	21	34
WMH/proteins (AD, MCI, controls)	46	51
WMH/proteins (AD)	30	37
Cognitive decline/mRNA (AD)	16	34
Cognitive decline/proteins (AD)	16	31

Table 24: Comparing actual versus ideal sizes of validation sets

A detailed elaboration of how the statistical power and ideal sample sizes were calculated can be found in the following sections after a brief introduction to conventions and definitions (Table 25).

Power of the models and sample sizes

Effect size (f^2) conventions		
$f^2 =$	0.02	Small effect
$f^2 =$	0.15	Medium effect
$f^2 =$	0.35	Large effect

Table 25: Effect size conventions

Here, the R^2 is used as effect size for all posthoc and a priori tests.

Statistical measure	*Description*
Effect size f^2	Effect size is a simple way of quantifying the difference between two groups that has many advantages over the use of tests of statistical significance alone. Effect size emphasises the size of the difference rather than confounding this with sample size.
α error probability	The rate of the type I error is called the size of the test and denoted by the Greek letter α (alpha). It usually equals the significance level of a test, which is the probability of rejecting the null hypothesis given that it is true.
F Distribution	The F distribution is the distribution of the ratio of two estimates of variance. It is used to compute probability values in the analysis of variance. The F distribution has two parameters: degrees of freedom (df) numerator and degrees of freedom denominator.
Numerator df	The number of degrees of freedom that the estimate of variance used in the numerator is based on.
Denominator df	The number of degrees of freedom that the estimate used in the denominator is based on.
Non-centrality parameter λ	The noncentrality parameter changes the shape of the F distribution in the analysis of variance, when there is an actual effect in the populations
Critical F	Critical value of the F-distribution
Power (1- β error probability)	1- β is the probability of rejecting a null hypothesis correctly when the alternative hypothesis is true. Sufficient sample sizes are required to achieve adequate power (sensitivity). Most researchers assess the power of their tests using $\pi = 0.80$ as a standard for adequacy.

Table 26: Description of statistical measures

The categorisation based on the correlation coefficient should be seen as a rule of thumb because the interpretation also depends on sample size and number of predictors. In general, the higher the correlation coefficient the stronger is the relationship between the variables.

Correlation threshold (Dancey and Reidy's, 2004)	
R	*Strength of correlation*
1	Perfect
0.7 – 0.9	Strong
0.4 – 0.6	Moderate
0.1 – 0.3	Weak
0	Zero

Table 27: Categorisation of correlation strength

3 Results

3.1 Detection of a panel of mRNA transcripts associated with WMH in AD|MCI|controls

To detect WMH-associated transcripts, in a mixed group of individuals with AD, MCI, and controls, a feature selection algorithm was applied which identified **17** significant mRNA transcripts (from a total of 6,282), in the training set. The detailed workflow is described in (Table 17). A summary of the significance levels and test statistics can be found in the appendix (Table 54). The panel of transcripts included **ALAS2** [aminolevulinate, delta-, synthase 2], **ARFGAP1** [ADP-ribosylation factor GTPase activating protein 1], **CC2D1B** [coiled-coil and C2 domain containing 1B], **CCDC90A** [mitochondrial calcium uniporter regulator 1], **CREB5** [cAMP responsive element binding protein 5], **EOMES** [eomesodermin], **FAU** [Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed], **FBXW4** [F-box and WD repeat domain containing 4], **HS.127310** [U2AF homology motif (UHM) kinase 1], **LILRA5** [leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5], **NAG18** [NAG18 mRNA], **NCAPD2** [non-SMC condensin I complex, subunit D2], **NRBP2** [nuclear receptor binding protein 2], **PNKD** [paroxysmal nonkinesigenic dyskinesia], **RRAGC** [Ras-related GTP binding C], **SHARPIN** [SHANK-associated RH domain interactor], and **ZNRD1A** [zinc ribbon domain containing 1].

To detect the best panel of mRNAs associated with WMH in a mixed group of AD, MCI, and controls, a linear regression function, with the option M5 enabled, was applied which included **12** transcripts from the 17 previously identified, in the model. In the training set, a weak correlation was found: the panel of 12 mRNA transcripts significantly predicted WMH n= 138, R^2= 0.43, $F(1,12)$= 7.83, P<0.001 (Table 28). In the cross-validated training set, there was little correlation n= 138, R^2= 0.15, and no correlation in an independent validation set n= 46/ R^2= -0.18; supplementary information can be found in the appendix (Table 55).

Linear regression of WMH-correlated mRNA transcripts n=138 (AD, MCI, and controls) in the training set

	Variable	*β*	*Error*	*T - value*	*P - v*alue
	Intercept	4896.5	549.2	8.916	4.92e-15 ***
1	**ALAS2**	8028.5	4911.4	1.635	0.1046
2	**ARFGAP1**	-8191.1	3792.9	-2.160	0.0327 *
3	**CCDC90A**	7635.5	4566.8	1.672	0.097 .
4	**CREB5**	7693.6	4672.0	1.647	0.10212
5	**EOMES**	5374.6	2431.6	2.210	0.0289 *
6	**FAU**	10756.5	5372.8	2.002	0.0474 *
7	**FBXW4**	-7732.5	4368.9	-1.770	0.0792 .
8	**LILRA5**	-1850.7	705.9	-2.622	0.0098 **
9	**NCAPD2**	6559.4	3119.1	2.103	0.0375 *
10	**PNKD**	10037.3	5308.6	1.891	0.061 .
11	**RRAGC**	7043.1	4012.5	1.755	0.0817 .
12	**SHARPIN**	-5684.4	3969.9	-1.432	0.1547

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residuals:

Min	1Q	Median	3Q	Max
-12410.1	-3599.2	-765.4	2152.2	28262.7

Residual standard error: 6233, Multiple R-squared: 0.43, Adjusted R-squared: 0.37
F-statistic: 7.829 on 12, p-value: 9.92e-11

Table 28: Model specification linear regression mRNA versus WMH in AD, MCI, and controls

Power and sample sizes WMH/mRNA (AD|MCI|CTL)

Post-hoc

The post-hoc test on the training set, with effect size 0.43, α error probability 0.05, n=138, 12 predictors, achieved a statistical power of **0.99** (Table 29). The validation set had zero effect size, thus no posthoc test can be performed.

A priori

To detect an effect size of 0.43 (like in the training), α error probability 0.05, power=0.99, 12 predictors, the required sample size of the validation set would have to contain **91** subjects (Table 29).

However, an acceptable power would be already achieved with 0.80. To detect an effect size of 0.43 (like in the training), α error probability 0.05, power=0.80, 12 predictors, the required sample size of the validation set would have to contain **52** subjects (Table 29).

F tests *– Linear multiple regression: Fixed model, R^2 deviation from zero*				
	Post hoc - achieved power of training set	**Post hoc - achieved power of validation set**	**A priori – required sample size of validation set for power of 0.99**	**A priori – required sample size of validation set for power of 0.80**
Input				
Effect size f^2	0.43	- - -	0.43	0.43
α error probability	0.05	- - -	0.05	0.05
Power (1- β error probability)	- - -	- - -	0.99	0.80
Total sample size	138	- - -	- - -	- - -
Number of predictors	12		12	12
Output				
Non-centrality parameter λ	59.34	- - -	39.13	22.36
Critical F	1.83	- - -	1.8	2.01
Numerator df	12	- - -	12	12
Denominator df	125	- - -	78	39
Total required sample size	- - -	- - -	**91**	**52**
Achieved Power (1- β error probability)	**0.99**	- - -	0.99	0.80

Table 29: Power and sample sizes WMH/mRNA (AD\|MCI\|CTL) models

3.2 Detection of a panel of mRNA transcripts associated with WMH in AD

To detect the best panel of mRNAs associated with WMH in a group consisting only of AD subjects (a subset of the previously used mixed AD, MCI, CTL group), a linear regression function, with the option M5 enabled, was applied which included **11** transcripts from previously identified 17 significant WMH-associated mRNA transcripts. The detailed workflow is described in (Table 18). In the training set, a strong correlation was found: the panel of 11 mRNA transcripts significantly predicted WMH n= 53, R^2= 0.75, $F(1,11)$= 11.25, P<0.001 (Table 30). There was little correlation in the cross-validated training set n=53, R^2= 0.26, and in an independent validation set n= 21/ R^2=

0.11; supplementary information can be found in the appendix (Table 56).

Linear regression of WMH-correlated mRNA transcripts n=53 (AD) in the training set					
Variable		*β*	*Error*	*T - value*	*P - v*alue
	Intercept	4528.9	700.1	6.469	9.31e-08 ***
1	**ALAS2**	13927.8	5001.5	2.785	0.0081 **
2	**ARFGAP1**	-10680.4	4259.7	-2.507	0.0162 *
3	**CCDC90A**	13316.6	6622.8	2.011	0.051 .
4	**EOMES**	6508.9	3966.2	1.641	0.1084
5	**FAU**	15046.7	7425.1	2.026	0.0493 *
6	**HS.127310**	18507.5	9831.0	1.883	0.0669 .
7	**LILRA5**	-1256.4	852.0	-1.475	0.148
8	**NAG18**	16711.4	6917.2	2.416	0.0202 *
9	**NCAPD2**	11510.8	3811.9	3.020	0.0043 **
10	**PNKD**	15898.8	6651.5	2.390	0.0215 *
11	**SHARPIN**	-8883.5	4347.1	-2.044	0.0475 *
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 Residuals: Min 1Q Median 3Q Max -12237 -2116 13 1732 8699 Residual standard error: 4538, Multiple R-squared: 0.75, Adjusted R-squared: 0.68 F-statistic: 11.25 on 11 DF, p-value: 3.291e-09					

Table 30: Model specification linear regression mRNA versus WMH in AD

3.2.1 Power and sample sizes WMH/mRNA (AD)

Post-hoc

The post-hoc test on the training set, with effect size 0.75, α error probability 0.05, n=53, 11 predictors, achieved a statistical power of **0.98**. The post-hoc test on the validation set, with effect size 0.11, α error probability 0.05, n= 21, 11 predictors, achieved a statistical power of **0.08** (Table 31).

A priori

To detect an effect size of 0.75 (like in the training), α error probability 0.05, power=0.98 (like in the training), 11 predictors, the required sample size of the validation set would have to contain **51** subjects.

However, an acceptable power would be already achieved with 0.80. To detect an effect size of 0.75 (like in the training), α error probability 0.05, power=0.80, 11 predictors, the required sample size of the validation set would have to contain **34** subjects (Table 31).

F tests *– Linear multiple regression: Fixed model, R^2 deviation from zero*				
	Post hoc - achieved power of training set	**Post hoc - achieved power of validation set**	**A priori – required sample size of validation set for power of 0.98**	**A priori – required sample size of validation set for power of 0.80**
Input				
Effect size f^2	0.75	0.11	0.75	0.75
α error probability	0.05	0.05	0.05	0.05
Power (1- β error probability)	- - -	- - -	0.98	0.80
Total sample size	53	21	- - -	- - -
Number of predictors	11		11	11
Output				
Non-centrality parameter λ	39.75	2.31	38.25	25.5
Critical F	2.03	3.1	2.04	2.26
Numerator df	11	11	11	11
Denominator df	41	9	39	22
Total required sample size	- - -	- - -	**51**	**34**
Achieved Power (1- β error probability)	**0.98**	**0.08**	0.98	0.80

Table 31: Power and sample size in WMH/mRNA (AD) models

3.3 Detection of a panel of proteins associated with WMH in AD|MCI|controls

To detect WMH-associated proteins, in a mixed group of individuals with AD, MCI, and controls, a feature selection algorithm was applied which identified **27** significant proteins (out of 1,016), in the training set. A summary of the significance levels and test statistics can be found in the appendix (Table 58). The panel of proteins included: **CXCL16** [chemokine (C-X-C motif) ligand 16], **TWEAK** [TNFSF12 - tumor necrosis factor (ligand) superfamily, member 12], **HPV E7 Type 16** [Human papillomavirus type 16 E7 Binding Protein/ TRIP13], **Macrophage maanose receptor**, **GDF11** [Growth Differentiation Factor 11/ BMP11], ALPL [**Alkaline phosphatase bone]**, **IgM** [CD40, -Tumor necrosis factor ligand], **FCG2B** [Fc fragment of IgG, low affinity IIb, receptor (CD32)], **Testican 2**, **CAMK2B** [calcium/calmodulin-dependent protein kinase II beta], **Chk2** [checkpoint kinase 2], **BCL2** [PPP1R20 -B-cell CLL/lymphoma 2], **Kallistatin** [SERPINA4], **STK16** [Serine/Threonine Kinase 16], **Vasoactive Intestinal Peptide**, **CNDP1** [carnosine dipeptidase 1 (metallopeptidase M20 family)], **Cathepsin H**, **MBD4** [methyl-CpG binding domain protein 4], **LCMT1** [leucine carboxyl methyltransferase 1], **SAA** [serum amyloid A1 cluster], **MMP12** [matrix metallopeptidase 12 (macrophage elastase)], **PH** [Cytohesin 2], **FAM107B** [family with sequence similarity 107, member B], **Coagulation Factor V** [Factor V Leiden], **C1QBP** [Complement Component 1, Q Subcomponent Binding Protein], **Esterase D** [ESD], and **GPC5** [glypican 5].

To detect the best panel of proteins associated with WMH, in a mixed group of AD, MCI, and controls, a linear regression function, with the option M5 enabled, was applied

which included **20** proteins from the 27 previously identified, in the model. The detailed workflow is described in (Table 19). In the training set, a strong correlation was found: the panel of 20 proteins significantly predicted WMH n= 136, R^2= 0.64, $F(1,20)$= 10.43, P<0.001 (Table 32). There was little correlation in the cross-validated training set n= 136, R^2= 0.18, and in an independent validation set n= 46, R^2= 0.09; supplementary information can be found in the appendix (Table 59).

Linear regression of WMH-correlated proteins n= 136 in AD, MCI, and controls in the training set					
Variable		*β*	*Error*	*T - value*	*P - value*
	Intercept	6111	500.1	12.220	< 2e-16 ***
1	**CXCL16soluble**	3820.9	2299.4	1.662	0.0993 .
2	**GDF11**	-6463.4	1964.9	-3.289	0.0013 **
3	**Alkaline phosphatase bone**	-1644.9	770.9	-2.134	0.035 *
4	**IgM**	-1217.2	828.7	-1.469	0.1446
5	**Testican 2**	- 4849.7	2698.6	-1.797	0.0749 .
6	**Chk 2**	6062.0	1096.6	5.528	2.05e-07 ***
7	**Bcl 2**	5828.8	3029.0	1.924	0.0568 .
8	**Kallistatin**	- 4397.3	2455.5	-1.791	0.076 .
9	**Vasoactive Intestinal Peptide**	-6794.1	4001.3	-1.698	0.0922 .
10	**CNDP1**	-1917.0	1047.6	-1.830	0.0699 .
11	**Cathepsin H**	3635.1	2430.2	1.496	0.1375
12	**MBD4**	-4192.8	2589.3	-1.619	0.1081
13	**LCMT1**	-6090.2	2182.7	-2.790	0.0062 **
14	**SAA**	761.0	337.7	2.254	0.0261 *
15	**PH**	1564.7	707.4	2.212	0.029 *
16	**FAM107B**	3433.2	1591.5	2.157	0.0331 *
17	**Coagulation Factor V**	6016.2	2090.8	2.878	0.0048 **
18	**C1QBP**	- 9031.1	4939.2	-1.828	0.0701 .
19	**Esterase D**	- 5234.8	1899.7	-2.756	0.0068 **
20	**GPC5**	3479.6	1753.7	1.984	0.0496 *
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					
Residuals:					

Linear regression of WMH-correlated proteins n= 136 in AD, MCI, and controls in the training set				
Variable	*β*	*Error*	*T - value*	*P - value*
Min 1Q Median 3Q Max -11849.2 -3203.0 -701.2 2345.8 16916.6 Residual standard error: 5621, Multiple R-squared: 0.64, Adjusted R-squared: 0.58 F-statistic: 10.43 on 20 DF, p-value: < 2.2e-16				

Table 32: Model specification of proteins versus WMH in AD, MCI, and controls

3.3.1 Power and sample sizes WMH/proteins (AD|MCI|CTL)

Post-hoc

The post-hoc test on the training set, with effect size 0.64, α error probability 0.05, n=136, 20 predictors, achieved a statistical power of **0.99**. The post-hoc test on the validation set, with effect size 0.09, α error probability 0.05, n=46, 20 predictors, achieved a statistical power of **0.11** (Table 33).

A priori

To detect an effect size of 0.64 (like in the training), α error probability 0.05, power=0.99, 20 predictors, the required sample size of the validation set would have to contain **80** subjects.

However, an acceptable power would be already achieved with 0.80. To detect an effect size of 0.64 (like in the training), α error probability 0.05, power=0.80, 20 predictors, the required sample size of the validation set would have to contain **51** subjects (Table 33).

F tests *– Linear multiple regression: Fixed model, R^2 deviation from zero*				
	Post hoc - achieved power of training set	**Post hoc - achieved power of validation set**	**A priori – required sample size of validation set for power of 0.99**	**A priori – required sample size of validation set for power of 0.80**
Input				
Effect size f^2	0.64	0.09	0.64	0.64
α error probability	0.05	0.05	0.05	0.05
Power (1- β error probability)	- - -	- - -	0.99	0.80
Total sample size	136	46	- - -	- - -
Number of predictors	20	20	20	20
Output				
Non-centrality parameter λ	87.04	4.14	51.20	32.64
Critical F	1.66	2.00	1.75	1.93
Numerator df	20	20	20	20
Denominator df	115	25	59	30
Total required sample size	- - -	- - -	**80**	**51**
Achieved Power (1- β error probability)	**0.99**	**0.11**	0.99	0.80

Table 33: Power and sample size calculation of WMH/proteins (AD, MCI, controls) models

3.3.2 KEGG pathways

In order to identify functional pathways in which the mRNAs from the mRNA expression analysis of cerebral white matter lesions play a role, the detected set consisting of ALAS2, ARFGAP1, CCDC90A, CREB5, EOMES, FAU, FBXW4, LILRA5, HS.127310, NAG18, NCAPD2, PNKD, RRAGC, and SHARPIN, was entered in the pathway analysis (sequence information see appendix).

One significant (p<0.05 Bonferroni corrected) pathway “Ribosome” was found (Table 35).

GO_id	Term	Number of Genes	P-value Bonferroni corrected
3010	Ribosome	8	<0.05

Table 34: Significant pathways from mRNA/WMH analysis

3.4 Detection of a panel of proteins associated with WMH in AD

To detect the best panel of proteins associated with WMH, in individuals with AD (a subset of the group as above, without MCI and controls), a linear regression function, with the option M5 enabled, was applied which included **13** proteins from the 27 previously identified. The detailed workflow is described in (Table 20). In the training set, a strong correlation was found: the panel of 13 proteins significantly predicted WMH n= 59, R^2= 0.76, $F(1,13)$= 10.85, P<0.001 (Table 35). There was a weak correlation in the cross-validated training set n= 59, R^2= 0.12, but little correlation in an independent validation set n= 30, R^2= 0.03 (Table 60).

Linear regression of WMH-correlated proteins n= 59 in AD in the training set					
Variable		*β*	*Error*	*T - value*	*P - value*
	Intercept	5666.5	775.1	7.311	3.53e-09 ***
1	**GDF 11**	-10119.7	2408.1	-4.202	0.0001 ***
2	**Testican 2**	-7568.9	4255.4	-1.779	0.0821 .
3	**Chk 2**	7750.3	1522.3	5.091	6.79e-06 ***
4	**Bcl 2**	22918.6	3749.7	6.112	2.13e-07 ***
5	**Kallistatin**	-6506.4	4056.0	-1.604	0.1157
6	**STK16**	15625.7	4912.3	3.181	0.0027 **
7	**CNDP1**	3598.6	1842.7	1.953	0.0571 .
8	**Cathepsin H**	11588.9	3486.3	3.324	0.0018 **
9	**SAA**	-950.7	500.9	-1.898	0.0641 .
10	**MMP 12**	8453.1	3845.0	2.198	0.0331 *
11	**FAM107B**	3119.8	1960.3	1.591	0.1185
12	**C1QBP**	-14941.8	6924.5	-2.158	0.0363 *

Linear regression of WMH-correlated proteins n= 59 in AD in the training set					
Variable		*β*	*Error*	*T - value*	*P - value*
13	**GPC5**	-15051.9	6065.6	-2.482	0.0169 *
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 Residuals: Min 1Q Median 3Q Max -10873.1 -3710.8 489.6 3037.7 8803.1 Residual standard error: 5159, Multiple R-squared: 0.76, Adjusted R-squared: 0.69 F-statistic: 10.85 on 13 DF, p-value: 6.272e-10					

Table 35: Model specification of proteins versus WMH in AD

3.4.1 Power and sample sizes WMH/proteins (AD)

Post-hoc

The post-hoc test on the training set, with effect size 0.76, α error probability 0.05, n=59, 13 predictors, achieved a statistical power of **0.99**. The post-hoc test on the validation set, with effect size 0.03, α error probability 0.05, n=30, 20 predictors, achieved a statistical power of **0.06** (Table 36).

A priori

To detect an effect size of 0.76 (like in the training), α error probability 0.05, power=0.99, 13 predictors, the required sample size of the validation set would have to contain **59** subjects.

However, an acceptable power would be already achieved with 0.80. To detect an effect size of 0.76 (like in the training), α error probability 0.05, power=0.80, 13 predictors, the required sample size of the validation set would have to contain **37** subjects (Table 36).

F tests *– Linear multiple regression: Fixed model, R^2 deviation from zero*				
	Post hoc - achieved power of training set	**Post hoc - achieved power of validation set**	**A priori – required sample size of validation set for power of 0.99**	**A priori – required sample size of validation set for power of 0.80**
Input				
Effect size f^2	0.76	0.03	0.76	0.76
α error probability	0.05	0.05	0.05	0.05
Power (1- β error probability)	- - -	- - -	0.99	0.80
Total sample size	59	30	- - -	- - -
Number of predictors	13	13	13	13
Output				
Non-centrality parameter λ	44.84	0.90	44.84	28.12
Critical F	1.94	2.40	1.94	2.18
Numerator df	13	13	13	13
Denominator df	45	16	45	23
Total required sample size	- - -	- - -	**59**	**37**
Achieved Power (1- β error probability)	**0.99**	**0.06**	0.99	0.81

Table 36: Power and sample sizes WMH/proteins (AD)

3.4.2 KEGG Pathways

In order to identify functional pathways in which the proteins associated with cerebral white matter lesions play a role, the detected set consisting of CXCL16 soluble, GDF11, Alkaline phospatase bone, lgM, Testican, CHK2, BCL2, Kallistatin, Vasoactive intestine Peptide, CNDP1, Cathepsin H, MBD4, LCMT1,SAA, PH, FAM107B, Coagulation Factor 5, C1QBP, Esterase D, GPC5, STK16, MMP12, was entered in the pathways analysis (sequence information see appendix). Seven significant (p<0.05 Bonferroni corrected) pathways were found: MicroRNAs in cancer, Cell cycle, p53 signaling pathway, Apoptosis, Amyotrophic lateral sclerosis (ALS), Colorectal cancer, PI3K-Akt

signalling pathway (Table 37).

GO_id	Term	Number of Genes	P-value Bonferroni corrected
5206	MicroRNAs in cancer	7	<0.05
4110	Cell cycle	6	<0.05
4151	PI3K-Akt signaling pathway	5	<0.05
4115	p53 signaling pathway	4	<0.05
4210	Apoptosis	4	<0.05
5014	Amyotrophic lateral sclerosis	3	<0.05
5210	Colorectal cancer	3	<0.05

Table 37: Significant pathways from proteins/WMH analysis

3.5 Detection of a panel of AD-cognitive decline-associated mRNA transcripts

To detect cognitive decline-associated transcripts, in individuals with AD, the rate of cognitive decline measures, previously generated (Chapter 1), were analysed together with mRNA transcripts. To eliminate irrelevant transcripts from the analysis a feature selection algorithm was applied which identified **27** mRNA transcripts (out of 6,282) in the training set. The detailed workflow is described in (Table 21). A summary of the significance levels and test statistics can be found in (Table Error: Reference source not found). The panel of transcripts included **ALS2CR2** [Amyotrophic Lateral Sclerosis 2 Chromosomal Region Candidate Gene 2], **BRD2** [bromodomain containing 2], **CCR6A** [chemokine (C-C motif) receptor 6], **CD81**, **CHMP6** [charged multivesicular body protein 6], **CNOT2** [CCR4-NOT transcription complex, subunit 2], **CTTN** [cortactin], **DCTN5** [dynactin 5 (p25)], **DUSP3** [dual specificity phosphatase 3], **GNLY** [granulysin], **GPAA1** [glycosylphosphatidylinositol anchor attachment 1], **HBA2A** [hemoglobin, alpha 2], **HLAB** [major histocompatibility complex, class I, B],

HS.445414 [chromosome 1 open reading frame 132], **KIAA0355** [uncharacterized protein], **PABPC1A** [poly(A) binding protein, cytoplasmic 1], **PPHLN1** [periphilin 1], **PRPF40A** [PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)], **PSCD1A** [cytohesin 1], **REPS2** [RALBP1 associated Eps domain containing 2], **RN7SL1** [RNA, 7SL, cytoplasmic 1], RNF7 [ring finger protein 7], **SLC5A6** [solute carrier family 5 (sodium/ glucose cotransporter), member 6], **TMEM137** [transmembrane protein 137], **TMEM59** [transmembrane protein 59], **VRK3A** [vaccinia related kinase 3], and **YIF1A** [Yip1 interacting factor homolog A (S. cerevisiae)].

To detect the best panel of mRNAs associated with the rate of cognitive decline, a linear regression function, with the option M5 enabled, was applied which included **12** transcripts from the 27 previously identified, in the model. In the training set, a very strong correlation was found: the panel of 12 mRNA transcripts significantly predicted the rate of cognitive decline n= 46, R^2= 0.86, $F(1,12)$= 17.01, P<0.001 (Table 38). There was a strong correlation in the cross-validated training set n= 46, R^2= 0.75, but only little correlation in an independent validation set n=16, R^2= 0.13; supplementary information can be found in the appendix (Table 60).

Linear regression of cognitive decline-correlated mRNA transcripts n= 46 in the training set					
Variable		*β*	*Error*	*T - value*	*P - value*
	Intercept	0.0037	0.0004	9.482	6.03e-11 ***
1	**CCR6A**	0.0071	0.0031	2.315	0.027 *
2	**CD81**	-0.0027	0.0009	-2.942	0.0059 **
3	**CTTN**	-0.0025	0.0024	-1.019	0.3157
4	**GNLY**	0.0032	0.0012	2.702	0.0108 *

Linear regression of cognitive decline-correlated mRNA transcripts n= 46 in the training set					
Variable		*β*	*Error*	*T - value*	*P - value*
5	**GPAA1**	-0.0048	0.0022	-2.214	0.0338 *
6	**HBA2A**	-0.0075	0.0027	-2.749	0.0096 **
7	**HLAB**	0.0052	0.0028	1.854	0.0727 .
8	**PABPC1A**	0.0034	0.001	3.330	0.0022 **
9	**PPHLN1**	0.0048	0.0021	2.328	0.0262 *
10	**PSCD1A**	-0.005	0.0022	-2.233	0.0324 *
11	**REPS2**	-0.006	0.002	-2.940	0.006 **
12	**RN7SL1**	0.0052	0.0018	2.898	0.0066 **
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 Residuals: Min 1Q Median 3Q Max -0.0037994 -0.0010043 -0.0002052 0.0010982 0.0035811 Residual standard error: 0.002, Multiple R-squared: 0.86, Adjusted R-squared: 0.81 F-statistic: 17.01 on 12 DF, p-value: 8.492e-11					

Table 38: Model specification rate of cognitive decline versus mRNA

3.5.1 Power and sample sizes cognitive decline/mRNA (AD)

Post-hoc

The post-hoc test on the training set, with effect size 0.86, α error probability 0.05, n= 46, 12 predictors, achieved a statistical power of **0.97**. The post-hoc test on the validation set, with effect size 0.13, α error probability 0.05, n=16, 12 predictors, achieved a statistical power of **0.06** (Table 39).

A priori

To detect an effect size of 0.86 (like in the training), α error probability 0.05, power=0.97 (like in the training), 12 predictors, the required sample size of the validation set would have to contain **47** subjects.

However, an acceptable power would be already achieved with 0.80. To detect an effect size of 0.86 (like in the training), α error probability 0.05, power=0.80, 12 predictors, the required sample size of the validation set would have to contain **34** subjects (Table 39).

F tests *– Linear multiple regression: Fixed model, R^2 deviation from zero*				
	Post hoc - achieved power of training set	**Post hoc - achieved power of validation set**	**A priori – required sample size of validation set for power of 0.97**	**A priori – required sample size of validation set for power of 0.80**
Input				
Effect size f^2	0.86	0.13	0.86	0.86
α error probability	0.05	0.05	0.05	0.05
Power (1- β error probability)	- - -	- - -	0.97	0.80
Total sample size	46	16	- - -	- - -
Number of predictors	12	12	13	13
Output				
Non-centrality parameter λ	39.56	2.08	40.42	29.24
Critical F	2.06	8.74	2.03	2.25
Numerator df	12	12	13	13
Denominator df	33	3	33	20
Total required sample size	- - -	- - -	**47**	**34**
Achieved Power (1- β error probability)	**0.97**	**0.06**	0.97	0.80

Table 39: Power and sample sizes cognitive decline/mRNA (AD)

3.5.2 KEGG Pathways

In order to identify functional pathways in which the mRNAs from the mRNA expression analysis of cognitive decline play a role, the detected set consisting of CCR6A, CD81, CTTN, GNLY, GPAA1, HBA2A, HLAB, PABPC1A, PPHLN1, PSCD1A, REPS2, RN7SL1, was entered in the pathway analysis (sequence information see appendix).

GO_id	Term	Number of Genes	P-value Bonferroni corrected
563	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	3	<0.05
5144	Malaria	3	<0.05
3013	RNA transport	3	<0.05

Table 40: Significant pathways from mRNA/cognitive decline analysis

3.6 Detection of a panel of AD-cognitive decline-associated proteins

To detect cognitive decline-associated proteins, in individuals with AD, the previously generated rate of cognitive decline measures (Chapter 1), were analysed together with proteins. To eliminate irrelevant proteins from the analysis a feature selection algorithm was applied which identified **15** proteins (out of 1,016) in the training set. The detailed workflow is described in (Table 22). A summary of the significance levels and test statistics can be found in (Table Error: Reference source not found). The panel of proteins included **IL2sRg** [Interleukin 2 receptor], **EPOR** [erythropoietin receptor], **Hemopexin**, **OX40 ligand** [TNFRSF4/ Tumor Necrosis Factor Receptor Superfamily, Member 4], **WIF1** [WNT inhibitory factor 1], **ENA78** [CXCL5/ chemokine (C-X-C motif) ligand 5], **Siglec9** [sialic acid binding Ig-like lectin 9], **IL19** [interleukin 19], **CRIS3** [HMGCR/ HMG-CoA reductase], **TFPI** [tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)], **CNDP1** [carnosine dipeptidase 1 (metallopeptidase M20 family)], **Thrombin** [coagulation factor II (thrombin)], **ENTP3** [ectonucleoside triphosphate diphosphohydrolase 3)], **IL6** [Interleukin 6], **BST1** [bone marrow stromal cell antigen 1], **CBX5** [chromobox homolog 5].

To detect the best panel of proteins associated with the rate of cognitive decline a linear regression function, with the option M5 enabled, was applied which included **8** proteins from the 15 previously identified, in the model. In the training set, a strong correlation was found: the panel of 8 proteins significantly predicted the rate of cognitive decline n=46, R^2=0.7, $F(1,12)$= 11.23, P<0.001 (Table 41). There was little correlation in the cross-validated training set n=46/ R^2= 0.35, and no correlation in an independent validation set n=16 / R^2= 0; supplementary information can be found in the appendix (Table 61).

Linear regression of cognitive decline-correlated proteins n= 46 in the training set					
Variable		*β*	*Error*	*T - value*	*P - value*
	Intercept	0.0031	0.0004	8.697	1.42e-10 ***
1	**EPOR**	0.0057	0.0022	2.633	0.0122 *
2	**OX40 ligand**	-0.0037	0.0021	-1.809	0.0784 .
3	**ENA78**	0.0097	0.0024	4.018	0.0003 ***
4	**IL19**	0.0064	0.0022	2.864	0.0068 **
5	**CRIS3**	-0.0166	0.0062	-2.705	0.0102 *
6	**TFPI**	0.0033	0.0011	3.116	0.0035 **
7	**IL6**	-0.0098	0.0049	-1.994	0.0534 .
8	**BST1**	-0.0031	0.0014	-2.228	0.0319 *
Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1 Residuals: Min 1Q Median 3Q Max -0.0065048 -0.0007046 0.0002001 0.0008854 0.0045911 Residual standard error: 0.00206, Multiple R-squared: 0.7, Adjusted R-squared: 0.64 F-statistic: 11.23 on 8 DF, p-value: 5.571e-08					

Table 41: Model specification cognitive decline versus proteins

3.6.1 Power and sample sizes cognitive decline/proteins (AD)

Post-hoc

The post-hoc test on the training set, with effect size 0.70, α error probability 0.05, n= 46, 8 predictors, achieved a statistical power of **0.97**. The validation set had zero effect size, thus no posthoc test can be performed.

A priori

To detect an effect size of 0.70 (like in the training), α error probability 0.05, power= 0.97 (like in the training), 8 predictors, the required sample size of the validation set would have to contain **45** subjects.

However, an acceptable power would be already achieved with 0.80. To detect an effect size of 0.70 (like in the training), α error probability 0.05, power=0.80, 8 predictors, the required sample size of the validation set would have to contain **31** subjects (Table 42).

tests *– Linear multiple regression: Fixed model, R^2 deviation from zero*				
	Post hoc - achieved power of training set	**Post hoc - achieved power of validation set**	**A priori – required sample size of validation set for power of 0.97**	**A priori – required sample size of validation set for power of 0.80**
Input				
Effect size f^2	0.70	- - -	0.70	0.70
α error probability	0.05	- - -	0.05	0.05
Power (1- β error probability)	- - -	- - -	0.97	0.80
Total sample size	46	- - -	- - -	- - -
Number of predictors	8	- - -	8	8
Output				
Non-centrality parameter λ	32.20	- - -	31.50	21.70
Critical F	2.2	- - -	2.21	2.40
Numerator df	8	- - -	8	8
Denominator df	37	- - -	36	22

Total required sample size	**- - -**	- - -	**45**	**31**
Achieved Power (1-β error probability)	**0.97**	- - -	0.97	0.81

Table 42: Power and sample sizes cognitive decline/proteins (AD)

3.6.2 KEGG pathways

In order to identify functional pathways in which the proteins associated with cognitive decline play a role, the detected set consisting of EPOR, OX40 ligand, ENA78, IL19, CRIS3, TFPI, IL6, BST1 entered in the pathways analysis (sequence information see appendix).

GO_id	Term	Number of Genes	P-value Bonferroni corrected
4630	JAK-STAT signaling pathway	10	<0.05
4060	Cytokine-cytokine receptor interaction	9	<0.05
4151	PI3K-Akt signaling pathway	6	<0.05
4917	Prolactin signaling pathway	4	<0.05
4640	Hematopoietic cell lineage	4	<0.05
5162	Measles	4	<0.05
5161	Hepatitis B	4	<0.05
5221	Acute myeloid leukemia	3	<0.05
4062	Chemokine signaling pathway	4	<0.05
5203	Viral carcinogenesis	4	<0.05
5220	Chronic myeloid leukemia	3	<0.05
4012	ErbB signaling pathway	3	<0.05
4066	HIF-1 signaling pathway	3	<0.05
5200	Pathways in cancer	4	<0.05

Table 43: Significant pathways from proteins/cognitive decline analysis

4 Discussion

4.1 Previously identified markers of cognitive decline

A number of blood biomarkers have been suggested such as inflammatory cytokines, oxidative stress, and thrombocytic/vascular proteins (O'Brian et al. 2003; Ho et al., 2005; Whiteley et al. 2009; Skoumalova et al. 2012). These studies investigated certain categories of proteins whereas the current study includes a large number of proteins (1,016) across various functional domains. Inflammation and vascular factors were associated with neurodegeneration and the pathogenesis of dementia (Schmidt et al., 2002). However, the question arises as to whether these inflammatory blood signatures indicate the presence of co-morbidities which is often likely in elderly individuals (Engelhart et al. 2004).

Inflammation markers of cognitive decline

Two large studies from the Netherlands, the Rotterdam study (3,874 subjects included in analyses), and the Leiden study (491 subjects included in analyses), investigated cognitive decline in old age (Schram et al., 2007). These studies can be of interest for comparison with the current study. All their analyses were adjusted for age, sex, and eduction because these factors have an impact on cognitive decline. The current study also tested for significance of these variates and included them if they returned

significant in the statistical model of AD disease progression. Schram et al. investigated elderly individuals without dementia whereas the current study examined AD patients in old age. Their study identified the cytokine IL-6 as associated with worse global cognition and executive function ($P<0.05$) as did the current study where IL-6 was associated with global cognitive decline ($P<0.05$). There could be a link to dementia onset in the elderly subjects under investigation in the Dutch studies: dementia develops over a long preclinical period so it could be a possible marker in the pre-dementia state in aged individuals, or it could be an indicator of a co-morbidity shared by the aged individual from the Dutch studies and the current study. Thus it is imperative to further investigate the function of such candidates before drawing any conclusions. IL-6 is possibly worthy of further investigation because markers of the earliest stage of cognitive decline are desperately needed before full-blown dementia symptoms occur.

Studies of blood markers which reported associations with cognitive decline exposed a number of limitations such as short-follow up time (which is also a concern in the current study), small subject pool, or the inclusion of only one candidate marker. Previously, many studies have shown minor associations between inflammatory signatures and cognitive decline but often with small effect size (Weaver et al., 2002; Teunissen et al., 2003; Yaffe et al., 2003; Dik et al. 2005). However, this does not necessarily mean that systemic inflammation is not a large contributor to cognitive decline. On the contrary, it is remarkable that inflammation markers can be identified in various populations with cognitive decline, and they are likely to be involved in

cognitive psychophysiology (Schram et al., 2007).

In conclusion, a number of studies, including the current study, show moderate associations between inflammatory proteins and cognitive decline but none of these proteins or protein panels are yet fully validated to predict the individual cognitive disease progression.

Some studies reported that APOE ε4 is an important factor for cognitive decline, others did not confirm this (Henderson et al., 1995; Nussbaum et al. 2003). In the current study, the influence of APOE ε4 on cognitive decline was also tested in the linear mixed model but the variable did not return significant. APOE ε4 is considered to contribute to cognitive decline by moderating inflammatory processes. The Rotterdam study found that IL-6 was associated with cognitive decline only in carriers of the APOE ε4 allele, whereas, the Leiden study found an association of IL-6 and cognitive decline in both carriers and non-carries but weaker in non-carriers (Schram et al., 2007). The proteins discovered could be due to chance only because they were not statistically controlled for multiple comparisons. A strength of the current study was a machine learning method which takes into account false positives in large datasets. Thus, proteins identified during feature selection were not merely found due to chance. However, running a post-hoc test on IL-6 alone against cognitive decline as the dependent variable, in the current study, reveals a model power of 0.47. A good model should at least have 0.80. However,

IL-6 together with the other identified proteins (EPOR, OX40 ligand, ENA78, IL19, CRIS3, TFPI, BST1) achieved a model power of 0.97 in the training set. The validation set was too small (consisting of only 16 AD patients) to confirm this model.

A number of studies demonstrated that large confluent WMH are associated with cognitive decline and dementia (Prins, 2015). Smaller lesions might have no or minor effects on cognition brain plasticity can compensate for these areas. WMH baseline volumes were also associated with more aggressive cognitive decline, 3 or more points over 6 months or 6 or more points over 12 months (Tosto et al., 2014).

Oxidative stress has an essential impact on the pathogenesis of AD and MCI and can be detected in the form of e.g. lipid peroxidation products in the blood (Skoumalova, 2011). These oxidative stress markers could potentially assist in the diagnosis of AD. However, the fact that they can also be detected in diabetes and cardiovascular diseases makes an AD specific diagnosis more difficult.

Oxidative stress, the overproduction of free radicals, has been described as a consequence of Ab misfolding in the presence of activated microglia. In AD patients, an enzyme NADPH oxidase which facilitates the production of free radicals has been detected.

Another factor is Fe2+ (iron) which can strongly contribute to free radical production, especially in the most aggressive from of lipid peroxidation.

4.2 Novel WMH-associated mRNA transcripts – potential biomarkers?

The following hypothesis was examined in a mixed group of individuals with AD, MCI, and cognitively normal individuals (AD|MCI|controls), and in a group containing only individuals with AD: "*there would be mRNAs and proteins in blood associated with WMH*". The aims pursued included to find such blood signatures which could serve as WMH blood markers in future when validated in independent cohorts. Overall, the findings suggest that WMH pathology affects both the blood's transcriptome and proteome. In total, **27** mRNA transcripts and **13** proteins were associated with WMH severity, in the feature selection which is a process for identifying statistically significant mRNAs (here from a total of 6,282) and proteins (here from a total of 1,016 proteins). Here, the feature selection was important to eliminate non-WMH-correlated mRNAs and proteins from the analysis. In addition, the identified features might be potential candidates for further investigation either individually or in combination.

4.2.1 A panel of WMH-associated mRNA transcripts in AD|MCI|controls

The key findings of this study is that WMH volumes at baseline were significantly associated with **17** single mRNA transcripts ($P<0.05$), and with a panel of **12** mRNAs (n= 138, $R^2= 0.43$, $P<0.001$) in individuals with AD, MCI, or cognitively normal individuals.

The results suggest that individually the transcripts are highly correlated with WMH, and that there is a correlation of the panel of mRNA transcripts with WMH volumes, in

the training set, but was not confirmed in the validation set. The limitation of these studies is that the validation sets were slightly underpowered.

The challenges of multi-center studies are also known in large-scale multi-center trials. In both cases studies often fail to achieve their recruitment targets, and even if there are many participants in total they may not undergo the same battery of assessments. This may result in an underpowered study, which, in turn, may lead to non-significant results that nevertheless do not rule out the possibility of important insights in the research question under investigation (Treweek et al., 2013).

Nevertheless, the identified mRNAs are worthy of further investigation because in previous studies the members of the panel, **ALAS2** (Xu et. al. 2010), **ARFGAP1** (Stafa et al. 2012), **CCDC90A** (Schutzer 2011), **CREB5** (Arezoo et al. 2011), **EOMES** (Patsopoulos et al. 2011), **FBXW4** (Soler-López et al. 2011), and **NCAPD2** (Li et al. 2009), have been found to be involved in diseases in which WMH burden is common, such as ischemic, neurodegenerative and demyelinating disorders. Other members of the panel have **not** been previously linked to WMH such as **PNKD**, **FAU**, **LILRA5**, **RRAGC**, **SHARPIN.**

Future work could aim to replicate these findings in larger, independent cohorts with special focus on the previously discovered and in this study suggested, mRNAs (ALAS 2, ARFGAP 1, CCDC 90A, CREB 5, EOMES, FBXW4, and NCAPD 2) and in addition the novel WMH-associated mRNAs (PNKD, FAU, LILRA 5, RRAGC, and SHARPIN) to investigate their potential as a WMH markers.

Of note, 5 WMH-associated mRNA transcripts were not included this panel but appeared in the feature selection were CC2D1B, HS.127310, NAG18, NRBP2, and ZNRD1A. NRBP2 was previously found by colleagues, in a related study, a blood gene expression marker of early AD, using the same cohorts (Lunnon et al. 2013).

4.2.2 *A panel of WMH-associated mRNA transcripts in AD*

The key findings of this study are that WMH volumes at baseline were significantly associated with **17** single mRNA transcripts ($P<0.05$), and with a panel of **11** mRNA transcripts (n=53, $R^2=0.75$, $P<0.001$), in individuals with AD.

The results suggest that individually the mRNA transcripts are highly correlated with WMH, and that there is a strong correlation of the panel of mRNA transcripts with WMH volumes, in the training set, but could not be confirmed in the validation set. The limitation of this study is that the validation set consists only of 21 subjects.

Compared to the previous analysis which investigated WMH-associated mRNA transcripts in AD|MCI|controls (n= 138, $R^2= 0.43$, $P<0.001$), the current analysis performed better by including only individuals with AD, (n= 53, $R^2= 0.75$, $P<0.001$).

In this panel of 11, only **ALAS2** and **NCAPD2** were highly significant ($P<0.01$). On an individual level, **EOMES** and **PNKD** ($P<0.001$), **ARFGAP1, CCDC90A, FAU, HS.127310, LILRA5, NAG18, NCAPD2,** and **RRAGC** ($P<0.01$), were highly significant.

Future work could aim to replicate these findings in larger, independent cohorts with special focus on the previously discovered and in this study suggested, mRNAs (ALAS

2, ARFGAP 1, CCDC 90A, CREB 5, EOMES, FBXW4, and NCAPD 2) and in addition the novel WMH-associated mRNAs (PNKD, FAU, HS.127310, LILRA 5, NAG 18, and RRAGC) to investigate their potential as a WMH marker.

4.2.3 Individual WMH-associated mRNA transcripts and their implication in biological processes or diseases

The identified mRNA transcripts represent genes or gene families which have been reported previously to be associated with (a) oxidative stress, (b) neuronal survival and differentiation, (c) multiple sclerosis, (d) dementia, and (e) cancer. These biological processes and diseases can be linked to white matter degeneration and demyelination.

(a) Oxidative stress related mRNA transcripts

A previous study which is of high relevance to the current work, has investigated RNA in blood of aged individuals and patients diagnosed with Alzheimer's disease. They found that, **ALAS2**, the rate-limiting enzyme of heme production, was over-expressed in subjects with high WMH volumes. A contribution of abnormal ALAS 2 expression to oxidative stress and cardiovascular disease has been suggested (Hall et al. 2009). They identified several other pathways related to abnormally high WMH burden such as inflammation, hormone, metabolic, and central nervous system signalling pathways, and genes linked to oligodentrocyte proliferation, axonal repair, long term potentiation and neurotransmission (Xu et. al. 2010). Their aim was to assess if there were significant differences in expression levels in two groups of patients: with high amount of WMH

and with small WMH volumes (Xu et. al. 2010). Their results suggest that WMH in normal ageing and WMH in Alzheimer's disease share a common molecular pathology.

Their subjects were drawn from the Alzheimer's Disease Center at University of California Davis an independent cohort compared to the current study. In both studies, WMH was associated with ALAS 2 expression. Thus, it can be concluded that there is most likely an association of WMH with ALAS 2.

(b) Neuronal survival and differentiation-related mRNA transcripts

CREB5, a central transcription factor, implicated in neuronal survival and differentiation, has been found within a list of SNPs significantly associated with deep WMH (Arezoo et al. 2011) (Table 44).

SNP	*Chromosome*	*Map position (bp)*	*Gene*	*Location*	*Trait associated*	*P-value*
rs2391665	7	28468319	CREB5	Intron	DWMH	6.1×10^{-6}
rs77993437	7	28474345	CREB5	Intron	DWMH	8.4×10^{-6}
rs7790864	7	28478625	CREB5	Intron	DWMH	8.3×10^{-6}
rs6462085	7	28479848	CREB5	Intron	DWMH	9.1×10^{-6}

Table 44: Deep WMH (DWMH)-related SNPs were located within genes implicated in neuronal survival and differentiation (CREB5) *(Arezoo 2011)*

(c) Multiple sclerosis-related mRNA transcripts

WMH burden in multiple sclerosis, a chronic inflammatory disease of myelin, is usually very high (Arezoo et al. 2011). A recent study identified new susceptibility alleles for multiple sclerosis: rs170934(T) at 3p24.1 (odds ratio [OR], 1.17; p = 1.6 × 10(-8)) near **EOMES** (Patsopoulos et al. 2011). Also in the current study, a link of EOMES with WMH is suggested.

CCDC90A has been suggested as a diagnostic biomarker for multiple scelerosis (Schutzer et al. 2011). Here, the link of CCDC90A with WMH is suggested.

Demyelination, a characteristic for individuals with Parkinson's a disease, has been associated with **ARFGAP1** (Schutzer 2011). **SLC44A4** may modulate (auto-) immune responses by TGF-beta (Stafa et al. 2012). Here, the link of ARFGAP1 and SLC44A4 with WMH is suggested.

In a reconstruction study of the herpes simplex virus immuno-cytochemical evidence demonstrated that HSV-containing organelles attached to the microtubules implicated the trans-Golgi network marker TGN46, a member of the TGN family (Fujita et al. 2012).

In the current study, **TGOLN2** is associated with WMH. In several cases of herpex simplex WMH have been observed (Lee et al. 2006).

(d) Dementia-related mRNA transcripts

Several of the identified mRNAs represent genes or gene families which have been previously linked to AD: **NCAPD2** (Li et al. 2009), **FBXW4** (Soler-López et al. 2011), and **NRBP2** (Lunnon et. al. 2013). A recent study has associated NCAPD2 with Parkinsons disease implicating SNPs in **NCAPD2** (rs7311174, $p = 0.05$; and rs2072374, $p = 0.01$) on chromosome 12p13 (Zhang et al. 2014). This locus contains genetic variants predisposed to AD (Lee et al. 2008). **UBAP1** is a risk factor for frontotemporal lobe dementia, and possibly ischemia (Rollinson et al. 2009).

(e) Cancer-related mRNA transcripts

Four of the WMH-associated mRNAs have been related to brain and other tumours, **NAG18** (Wimmer et al. 1999)**, FAU** and **RRAGC** (Mirrakhimov et al. 2013), and **ZNRD1A** which regulates BCL2 (Guo et al 2008). Enhanced BCL2 expression was accompanied by increased apoptosis in CD4+ T cells and natural killer cells of AD patients (Humpel et al. 2011). BCL2 was also found in another AD biomarker study who investigated the same cohort as in the current study (Hye et al. 2014). **FAU** is differentially expressed in autism, and glioblastoma, and **RRAGC** has been found in connection with brain neoplasms, and cholangiocarcinoma (Mirrakhimov et al. 2013). **PNKD** – mutations in this gene cause paroxysmal dyskinesia, and alter protein cleavage and stability (Shen et al. 2011).

4.2.4 A panel of WMH-associated mRNA transcripts and their implication in biological pathways

One significant functional pathway, 'Ribosome', in which the mRNAs from the expression analysis of WMH play a role, was detected. The ribosomal transport system can selectively move macromolecules between the nucleoplasm and cytoplasm and may also respond to differing growth and environmental conditions (Aitchison 2000).

A number of mutations can affect ribosomal proteins such as translation initiation factors and their regulators. Translation factors support particular steps of translational activities, and such factors are necessary for translation initiation in mammalian cells. For instance, eIF2, transports the initiator methionyl-tRNA (Met-tRNAiMet) to the ribosome to locate the start codon19. eIF2B is the first cytosolic translation initiation

factor to be identified as being related to human disease. Mutations in any of the genes for the eIF2B subunits, for example, EIF2B1–5, cause a severe autosomal recessive neurodegenerative disorder called 'leukoencephalopathy with vanishing white matter' (Van der Knaap, et al 2002).

The fact that the functional pathway 'Ribosome' was identified could indicate that particular ribosomal activities are associated with increased WMH.

4.3 Novel WMH-associated proteins – potential biomarkers?

4.3.1 A panel of WMH-associated proteins in AD|MCI|controls

The key findings of this study is that WMH volumes at baseline were significantly associated with **27** single proteins (25 proteins at $P<0.05$ significance level; 2 proteins at $P<0.1$ significance level), and with a panel of **20** proteins (n= 136, $R^2= 0.64$, $P<0.001$) in patients with AD, MCI, or cognitively normal individuals.

The results suggest that individually most of the proteins are highly correlated with WMH, and that there is a strong correlation of the panel of proteins with WMH volumes, in the training set, but this could not be confirmed in the validation set.

The limitation of these studies is that the validation sets were slightly underpowered.

Nevertheless, the identified proteins are worthy of further investigation because some members of the panel, **SAA** (Chung et al. 2000), **LCMT1** (Miyamoto et al. 2013), **Esterase D (**Hodler et al. 2012) have been previously associated with white matter damage. There were also individual proteins, identified by feature selection, which have

been previously linked to white matter degeneration, such as **CathepsinH** (Han et al. 2003) (Manningen et al 2014).

In this panel of 20, **CHK2** (P<0.001), **GDF11**, **LCMT1**, **F5**, and **Esterase D** (P<0.01), were highly significant. On an individual level, **GDF11**, **CHK2**, and **CathepsinH**, **FAM107B**, **GPC5**, and **PH** were highly significant (P<0.001).

Future work could aim to replicate these findings in different, independent cohorts with special focus on the previously discovered and in this study suggested, proteins (SAA, LCMT1, Esterase D, and Cathepsin H), and in addition the novel WMH-associated proteins (GDF 11, CHK 2, FAM107B, GPC5, and PH) to investigate their potential as a WMH/vascular disease marker.

4.3.2 A panel of WMH-associated proteins in AD

The key findings of this study is that WMH volumes were significantly associated with **27** single proteins (25 proteins at P<0.05 significance level; 2 proteins at P<0.1 significance level), and with a panel of **13** proteins (n= 59, R^2= 0.76, P<0.001) in individuals with AD.

The results suggest that individually most of the proteins are highly correlated with WMH, and that there is a strong correlation of the panel of proteins with WMH volumes, in the training set, but this could not be confirmed in the validation set.

The limitation of these studies is that the validation sets were slightly underpowered.

Nevertheless, the identified proteins in the panel are worthy of further investigation: **SAA** has been previously associated with white matter damage (Chung et al. 2000),

Testican2 with bone metabolism diseases (possible underlying genetic component for severe WMH) (Hausser et al. 2004), and **CathepsinH** (Han et al. 2003).

In this panel of 13, **GDF11**, **CHK2**, and **BCL2** ($P<0.001$), **STK16** and **CathepsinH** ($P<0.01$) were highly significant. On an individual level, **GDF11**, **CHK2**, and **CathepsinH**, **FAM107B**, **GPC5**, and **PH** were highly significant ($P<0.001$).

Future work could aim to replicate these findings in different, independent cohorts with special focus on the previously discovered and in this study suggested, proteins (SAA, Testican 2, and Cathepsin H), and in addition the novel WMH-associated proteins (GDF 11, CHK 2, BCL 2, STK16, FAM107B, GPC5, and PH), to investigate their potential as a WMH marker. Such a marker could be important for patient selection in clinical trials, if MRI images are not available the blood marker could provide the information on the white matter status of the patients, and on possible vascular co-morbidities. It could be that AD patients are required in trials who do not have vascular pathology, signs of multiple sclerosis or Parkinson's disease. In future, if the proteins could be confirmed in larger cohorts they could serve as WMH/vascular disease biomarkers.

4.3.3 Individual WMH-associated proteins and their implication in biological processes or diseases

Some of the proteins were reported in previous studies. Many of them were linked to demylinating diseases such multiple sclerosis and leucoencephalocies, rare bone diseases with WMH, bone and cartilage abnormalities, cancer, neurodegeneration, age, and AD.

(a) <u>White matter damage, multiple sclerosis, atherosclerosis, vascularisation</u>

There is evidence that **SAA** plays a role in the neuronal loss and white matter damage occurring in AD and in multiple sclerosis. Chung and colleagues used immuno-histochemical methods for localizing the injury specific apolipoprotein, acute phase serum amyloid A (A-apoSAA), and have demonstrated that affected regions in brains of individuals with AD and multiple sclerosis appeared intense, staining for A-apoSAA in comparison to an unaffected region and non-AD/multiple sclerosis brains (Chung et al. 2000). A recent study linked SAA to ischemic lesions in neonatals where SAA expression is increased in response to hypoxia leading to a greater risk of mortality (Hamed et al 2011). Another protein, linked to white matter lesions is **C1QBP** which is found in viral diseases caused by mosquitoes, for example Chagas disease. One of the pathological symptoms that is observed after infection with the virus are WMH. In the current study a possible association of WMH and C1QBP has been suggested. Regulatory transcription factor binding sites in the **LCMT1** gene promoter include CREB which is identified as an WMH-associated mRNA transcript in the current study. cAMP response element-binding (CREB) is known to mediate oligdentrocyte differentiation interfering with renewal of white matter (Chung et al. 2000).

EsteraseD is associated with optic neuritis and can be observed in 87% of individuals with multiple sclerosis. The disease is affecting the optic nerve and can be accompanied by ischemic white matter lesions or result from ischemia (Miyamoto et al. 2013). The association of WMH and Esterase D is here suggested. **Vasoactive intestal polypetide** has emerged as anti-inflammatory candidate to treat multiple sclerosis because of its abundance in the immune system (Hodler et al. 2012).

Progressive brain volume loss and white matter degeneration has been detected in MRI of mice with Unverricht-Lundborg type progressive myoclonus epilepsy. Disease-causing mutations in CSTB are currently known which lead to reduced expression of the cystatin B, ubiquitously expressed inhibitor of lysosomal cysteine cathepsins B, H, K, L, and S. (Manningen et al 2014). **CathepsinH** has also been detected in connection with atherosclerotic lesions (Tan & Waschek 2011). A link between cathepsin H and increased WMH is present in the current study.

Many of the proteins have been previously associated with cerebro-vascular events. **GDF11**, a member of the TGF-β family, facilitates neurogenesis and can possibly improve vascularisation in the brain (Han et al. 2003). GDF11 has been associated with accelerated rate of cognitive decline (P<0.05) (Sattlecker et al. 2014).

Elevated levels of **Alkaline phosphatase** can be found in patients with bone -conditions and -tumors, and are associated with vitamin D and calcium deficiency. Patients with bone disease and leucoencephalocies have usually high WMH burden. **IgM** plays an important role in immune responses, increased activation of inflammatory molecules correlates with a more demyelination, axonal loss, and WMH (Katsimpardi et al. 2014).

Coagulation Factor V is a gene encoding for “Factor V“ which is a protein of the coagulation system. Contrary to many other coagulation factors, it is not active as an enzyme but as a cofactor. Factor V mutations predispose for thrombosis. Previous literature and here F5 transcripts suggest that WMH arise in the context of vascular events. **CYTH1** expression occurs in embryonic tissues and stem cells, myelinating oligodendrocyte cells in the forebrain white matter. The gene is linked to cascades

involving the decrease of collagen-induced vascular permeability and arthritis in mice (Perini et al. 2006).

(b) Oxidized plasma proteins

The modification of proteins by reactive oxygen species plays an important role in AD.

Previously, specific oxidized proteins in blood plasma of AD subjects have been observed with a two- to sixfold greater specific oxidation index compared to controls, including isoforms of *fibrinogen γ-chain* precursor protein and of *α-1-antitrypsin precursor*. It has been postulated that these oxidized proteins could be biomarkers for AD (Zhu et al. 2000).

In the current study, a chemokine (C-X-C motif) ligand 16 has been associated with WMH. Previously, the chemokine which specifically binds to OxLDL (oxidized low density lipoprotein) has been implicated in the pathophysiology of atherogenesis, oxidative damage, WMH, and autoimmune responses in multiple sclerosis (Choi et al. 2002) (Fukumoto et al. 2004). Here, the association of CXCL16 with WMH is suggested.

In the current study, serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), which inhibits human amidolytic and kininogenase activities of tissue kallikrein, has been associated with WMH. Kallistatin is hypothesised to be linked to initiation of neuro-degeneration due to the neurotrophic properties of the proteins of the SERPINA family (Opsahl & Kennedy 2005; Horstman et al. 2010; Haider et al. 2011).

Coagulation factor II, which converts fibrinogen to fibrin and activates other factors V, VII, VIII, XIII, with functions in blood homeostasis, inflammation, and wound healing, is associated with cognitive decline in the current study.

CXCL16, Kallistatin, and **BCL2** also appeared in a list of biomarkers suggested for non-small cell lung-cancer identified in serum and tissue samples (Opsahl & Kennedy 2005).

CNDP1 has been associated with paraplegia, a disorder characterized by degeneration of the corticospinal tracts and posterior column of the spinal cord with more prominent WMH (Hourani et al. 2009). Sattlecker and colleagues found CNDP1 to be significant in the classification of AD *versus* controls subjects (Sattlecker et al 2014).

(c) Bone metbolism/Bone diseases

Members of the **Testican** family may participate in the regulation of matrix turnover in cartilage (Hausser et al. 2004). Increased WMH volumes have been detected in individuals with abnormal bone and cartilage metabolism with an underlying genetic component. Mutations in one of two genes encoding different subunits of a receptor signalling complex, TYROBP and TREM2, are the suspected culprits (Bock 2013). There is potential to draw an overarching picture to mechanisms in AD involving these two genes (Hausser et al. 2004).

(d) Cancer-related, age-related, AD-related

Variants of **CHK2** are associated with various sorts of cancer, and enhances tau

pathology in model organisms (Jonsson et al. 2013). Members of the CHK family are implicated in the cell cycle control (Iijima-Ando et al. 2010). In the current study Chk is somehow associated with elevated WMH burden. CHK2 been associated with entorhinal volume (P<0.05) in AD (Sattlecker et al. 2014). **CAMK2B** has been significant in a study classifying AD *versus* controls (P<0.05) (Sattlecker et al. 2014). **STK16** and **C1QBP** have been linked to cognitive decline (P<0.05) in AD (Sattlecker et al. 2014). **CNDP 1** and **MMP12** were found to be significant in a classifier AD *versus* controls (Sattlecker et al 2014). **TWEAK** was found to be significant in MCI versus converters with MCI to AD (P<0.05) (Sattlecker et al. 2014). **MBD4** has not been previously related to WMH however, here a correlation was found. **FAM107B** is a locus on chromosome 10 showing strong association with late-onset AD (Grupe et al. 2006). Later studies show that FAM107B expression is observed in tumour development and proliferation (Nakajima et al. 2012).

4.3.4 A panel of WMH-associated proteins and their implication in functional pathways

(a) MicroRNAs in the cancer pathway

MicroRNAs are small (ca. 18 - 25 nucleotides) noncoding single-stranded RNA molecules regulating gene expression and modulating the stability and translational efficiency of mRNAs. Recently, several studies have shown that microRNAs modulate the response to ischemia reperfusion injury and regulate expression in a number of important molecules involved in cell survival and apoptosis (Di et al. 2014). In the current study, seven mRNAs were implicated in a functional pathway that includes

microRNAs in cancer. It could be that some of them also modulate processes in response to ischemic injuries.

(b) Cell cycle and PI3K-Akt signaling pathway

In diseases with WMH, such as leukodystrophies, periventricular leucomalacia, or multiple sclerosis, the hypomyelination or the remyelination failure by oligodendrocyte progenitor cells implicates errors in the sequence of processes that usually occur during development when progenitors proliferate, migrate through the white matter, contact the axon, and differentiate into myelin-forming oligodendrocytes (Nguyen, 2006). Many events underlie the progressive deterioration which is specific to demyelination. Cell cycle regulating factors that coordinate progenitor cell division and the onset of differentiation, i.e. the initial steps of oligodendrocyte lineage progression, are essential in WMH diseases. In the current study, 6 proteins were implicated in the cell cycle pathway, and 5 proteins in the PI3K-Akt signaling pathway (another important pathway for cell cycle regulation) which could indicate that some early cell developmental processes in individuals with WMH could be abnormal.

(c) Apoptosis and p53 signaling pathway

Four proteins were implicated in the p53 and apoptosis pathways. Apoptosis plays a crucial role in cancer (Haupt 2003), and neurodegenerative diseases to eliminate excess, damaged or infected cells (Ghavami et al. 2014). Cerebrovascular WMH are accompanied by apoptosis of oligodendroglia and have been suggested to appear as a consequence of chronic cerebral ischemia (Tomimoto et al. 2003). It could be that the four identified proteins reflect such apoptotic processes in this patient population.

(c) Amyotrophic Lateral Sclerosis and Colorectal cancer pathway

WMH have been found in patients with Amyotrophic Lateral Sclerosis attributed to disease of the gastrointestinal tract (Brown et al. 2009). The the three proteins which were implicated in the significantly enriched pathway 'Amyotrophic Lateral Sclerosis' play also a role in Colorectal cancer. It may be that there is an association of intestinal diseases and WMH.

4.4 Novel AD cognitive decline-associated mRNA transcripts – potential biomarkers for disease progression?

4.4.1 A panel of AD cognitive decline-associated mRNA transcripts

The key findings of this study are that the individual rates of cognitive decline (cognitive slopes) were significantly associated with **27** single mRNA transcripts (25 mRNAs at $P<0.05$; 2 mRNAs at $P<0.1$ significance level), and with a panel of **11** mRNA transcripts (n=46, $R^2=0.86$, $P<0.001$), in individuals with AD, in the training set but only little correlation was found in an independent validation set (n=16, $R^2=0.13$). The results suggest that individually most of the mRNAs are highly correlated with the cognitive slopes, and that there is a strong correlation of the panel of mRNAs with the slopes, in the training set, but this could not be confirmed in the validation set.

The limitation of these studies is that the validation sets were slightly underpowered.

Nevertheless, the identified mRNAs in the panel are worthy of further investigation: **TMEM137** (Ehlert et al. 2007), **SLC5A6** (Shah et al. 2012), **REPS2** (de Ligt et al. 2012) have been previously associated with cognitive function or decline.

In this panel, **CD81**, **HBA2A**, **PABPC1A**, **REPS2**, and **RN7SL1** were highly significant (P<0.01). On an individual level, **ALS2CR 2** and **SLC5A6** (P<0.001), **CCR6A**, **CNOT2**, **CTTN**, **GNLY**, **GPAA1**, **HBA2A**, **KIAA0355**, **PABPC1A**, **REPS2**, **RN7SL1**, and **TMEM59** were highly significant (P<0.01).

Future work could aim to replicate these findings in larger, independent cohorts with special focus on the recently discovered, and in this study with cognitive decline associated mRNAs: TMEM 137, SLC 5A 6, REPS 2, and in addition the novel AD-cognitive decline associated mRNAs, ALS2CR 2, CD 81, HBA 2A, PABPC 1A, RN7SL 1, CCR 6A, CNOT 2, CTTN, GNLY, GPAA 1, HBA 2A, KIAA0355, PABPC 1A, RN7SL 1, and TMEM 59.

4.4.2 Individual AD cognitive decline-associated mRNA transcripts and their implications in biological processes or diseases

The mRNA transcripts identified in the current study represent genes or gene families which have been reported previously to be associated with (a) memory, (b) bipolar disorder, (c) Huntington's disease, (d) Alzheimer's disease, (e) brain metabolism, (f) lipid metabolism, (g) apoptosis, (h) sickle cell anemia, and (i) cell signalling. In these diseases and conditions symptoms such as cognitive impairment or rapid cognitive decline play a vital role. One transcript was found, **KIAA0355**, which is yet uncharacterised.

(a) Memory

Hippocampal **CTTN** levels, phosphorylation, and cognitive processing have been observed to be altered during learning and long-term potentiation (Davis et al 2006). The transcription of several DUSP genes, including **DUSP3,** is induced by ERK 1/2. The MAPK/ERK-CREB-Zif268 cascade contributes to the consolidation of long-term memory (Ekerot et al 2008).

DUSP3 has been suggested by Sattlecker and colleagues to be associated with cognitive decline in AD (P<0.05) (Sattlecker et al 2014). CREB was associated with WMH in the current study. **VRK3A** is a binding partner of DUSP3. They both play a role in the ERK pathway important for cognitive functions such as learning and memory (Kang & Kim 2008) (Krab et al. 2008).

(b) Mental disorder / Bipolar disorder

DCTN5 has been investigated in connection with psychiatric cognition disorders such as bipoloar disorder and is likely to be involved in regulating neural network physiology (MacLaren et al 2011). **YIF1A** has been linked with bipolar disorder in a GWAS analysis (Sklar et al 2012). Patients with bipolar disorder often have symptoms such as cognitive impairment of memory, attention and executive functions (Goodwin et al 2008). **PSCD1A** interacts with ARF6 which is involved in mental disorders (Myers et 2012). **CCR6** is a chemokine receptor that plays a crucial role in number of autoimmune diseases. It has been identified in human atherosclerotic plaques, but its functional role in atherogenesis remains unknown. Wan and colleagues found that genetic deletion of CCR 6 in ApoE−/− mice significantly reduced atherosclerosis and macrophages in aortas (Wan et al. 2012). A recent study found that CCR 6 exerted effects on cognition in

mice; higher locomotor activity and lower anxiety has been observed, and they were less interested in new social contacts (Jaehne et al 2014).

(c) Huntington's disease

Another study presented an interaction of a Huntingtin-associated protein with a member of the **DCTN** family. Huntingtin's is characterised by vast cognitive and behavioural changes (Paulsen 2011). **PPHLN1** is associated with aggregation and deposition of pathogenic proteins in Huntington's disease (Tavanez et al. 2005). Also **PRPF40A** is linked to the development of Huntington's disease with implications for nuclear toxicity and neurodegeneration (Scappini et al. 2007).

(d) Alzheimer's disease

TMEM59 modulates glycosylation, cell surface expression, and secretion of the amyloid precursor protein in AD (Ullrich et al. 2010). p97, a protein found in reactive microglia and linked to amyloid plaques, has been described as an AD marker (Ujiie et al. 2002), and is attached to the cell surface by **GPAA1** (Eisenhaber et al. 2014). **EPOR** was significantly associated with hippocampal volume in AD (Sattlecker et al), and with cognitive decline in the current study.

(e) Brain metabolism

Brain metabolism contributes to cognitive decline in AD and is likely influence the severity of the disease. Glucose transporters such as **SLC5A** play a role in AD and diabetes for the maintenance of proper neuronal functioning including cognition (Shah et al. 2012).

(f) Lipid metabolism

TMEM137 linked to Farber lipogranulomatosis, a rare inherited condition involving lipid metabolism in the body. In affected individuals intellectual disability can be observed and severe neurologic deterioration (Ehlert et al. 2007). **CD81** interacts with galanin and vasopressin and is affecting behaviour and cognitive ageing, and can be found in pathways of immune responses and lipid metabolism (Kadish et al. 2009).

(g) Apoptosis

GNLY is involved in mediating processes which can induce apoptosis in brain cells and play a role in cognitive impairment (de Ligt et al. 2012).

(h) Sickle cell anaemia

HBA2A increased levels were found in sickle cell anemia (Petranovic et al. 2014). Sickle cell anemia has been linked to impaired cognitive function in adults and is likely to have a large negative effect on the brain (Ballas et al. 2010).

(i) Cell signalling

REPS2 is part of a protein complex that regulates the endocytosis of growth factor receptors and has been related with intellectual disability (de Ligt et al. 2012).

4.4.3 A panel of AD cognitive decline-associated mRNA transcripts and their implications in biological processes or diseases

(a) Glycosylphosphatidylinositol (GPI)-anchor biosynthesis pathway

About 100 diseases have recently been identified as being associated with alterations in

the glycosylation pathways. For instance, defects in the GPI-anchor pathway cause muscular dystrophies (MD) (Ng & Freeze, 2014). Children with MD show cognitive impairments, including problems with receptive language, expressive language, visuo-spatial skills, fine-motor skills, attention, and memory skills. It could be that in the AD patients of the current sample some glycosylation related changes occur.

(b) Malaria

Children who had Malaria showed impairments in speech and language tasks (higher level language functions, vocabulary, pragmatics, phonology) and cognition (non verbal functioning) (Fernando et al. 2010). In the current study, 3 cognitive decline-associated mRNAs were implicated in the functional Malaria pathway.

(c) RNA transport

Differences in gene expression of neural processing pathways could contribute to individual differences in maintenance or deterioration of cognitive function. There has been evidence for up-regulation of actin-related processes and down-regulation of translation, RNA processing and localization, and vesicle-mediated transport in individuals with cognitive decline (Wilmot et al 2008). Transcriptional differences in AD patients compared to normal individuals have been reported (Blalock et al. 2004).

4.5 Novel AD cognitive decline-associated proteins – potential blood biomarkers for AD progression?

4.5.1 A panel of AD cognitive decline-associated proteins

The key findings of this study are that the individual rates of cognitive decline (cognitive slopes) were significantly associated with **15** single proteins (13 proteins at $P<0.05$; 2 proteins at $P<0.1$ significance level), and with a panel of **8** proteins (n=46, $R^2=0.7$, $P<0.001$), in individuals with AD, in the training set but this could not be confirmed in the validation set. In fact, there is overtraining in the training set. Future work should include testing a number of random training and test splits. The limitation of this study is that the validation set includes only 16 subjects. It was expected that no validation can be achieved with this small set. However, the results suggest that individually most of the identified proteins are highly correlated with the cognitive slopes and that there is a strong correlation of the panel of proteins with the slopes, in the training set.

The limitation of these studies is that the validation sets were slightly underpowered.

Nevertheless, some of the identified proteins in the panel are worthy of further investigation: **IL6** (Athilingam et al. 2012), **TFPI** (Kim et al 2013) (Sattlecker et al 2014), **IL19** (Sattlecker et al 2014) have been previously associated with cognitive decline or cognitive functioning. Also some of the proteins not in the panel, but identified in the feature selection, such as **IL2** (Meola et al 2013), **ENTPD3** (Sattlecker et al 2014), have been linked to cognitive deterioration.

In this panel, **ENA78**, **IL19**, and **TFPI** were highly significant (P<0.01). On an individual level, **TFPI** (P<0.001), **IL2sRg**, **ENA78**, **Thrombin**, and **BST1**, were highly significant (P<0.01).

Future work could aim to replicate these findings in different, independent cohorts with special focus on the recently discovered and in this study suggested proteins by Sattlecker and colleagues **TFPI**, **IL19**, **ENTPD 3**, **IL2sRg** (Sattlecker et al. 2014), and **IL6** (Athilingam et al. 2012), and on the novel proteins **ENA78**, **Thrombin**, and **BST1** here first associated with cognitive disease progression.

4.5.2 Individual AD cognitive decline-associated proteins and their implications in biological processes or diseases

The identified AD-cognitive decline-associated proteins are implicated in pathways of (a) inflammation/the immune system, (b) Alzheimer's disease, and (c) Parkinson's disease.

(a) Inflammation/the immune system

IL2 enhances transmission of dopamine, has effects on the immune system and has led to neuro-degeneration in mice and worse cognitive performance in cancer patients. Treated with IL 2 patients show cognitive changes in spatial memory and planning. IL 2 can also increase the activity of its precursor enzyme, choline acetyltransferase which alters cognitive performance in mice. IL 2 is present in elevated levels in the hippocampal areas of post-mortem AD patients (Meola et al. 2013). Sattlecker and

colleagues have found **IL2sRg** to be significant in the classification of AD *versus* controls (Sattlecker et al. 2014).

IL19 can bind the IL 20 receptor complex and lead to the activation of the signal transducer and activator of transcription 3 (STAT3) which is implicated in immune processes and has shown anitinflammatory properties in vascular diseases (England et al 2012).

IL6 has shown an inverse association of plasma IL-6 with attention/working memory (Athilingam et al. 2012). IL-6 has also been suggested to be a biomarker of inflammation (Fornage et al. 2008). Cytokines such as IL 6 have been implicated in in neuropsychiatric disorders, and elevated levels of this cytokine have been associated with cognitive impairment and heart failure (Fishman 2012).

OX40/OX40L regulates immunity by mediating propagation of T-cell responses and is linked to deterioration of immune function with in advanced age (Xia et al. 2000). Sattlecker and colleagues found OX40 and WIF in a classification AD *versus* controls and **IL19** in MCI *versus* MCI converting to AD (Sattlecker et al 2014).

(b) Alzheimer's disease

Hippocampal **EPOR** was elevated in individuals with AD and MCI compared to normal controls. There were heterogenous patterns of EpoR distributions in the cortex and hippocampus. Upregulation of EpoR in these regions could represent neuroprotective processes in the pathogenesis of late onset AD (Assaraf et al. 2007). **CRIS3** Rate-limiting enzyme for cholesterol synthesis is used in drugs for AD patients (statins)

(Assaraf et al. 2007). **TFPI** has recently been suggested as a candidate marker of endothelial damage in AD, and is associated with cognitive function in AD (Kim et al 2013). Chemokines as mediators of inflammation have been implicated in the pathophysiology of neurodegenerative diseases. The presence of the chemokine receptor CXCR3 and its ligands could play a role in AD. Findings of Sattlecker and colleagues are here suggested: **TFPI** and **ENTPD3** were significantly associated with the rate of cognitive decline in AD (Sattlecker et al 2014). **CBX5** identified to be associated with cognitive decline in the current study was linked to right hippocampal volume in the study of Sattlecker and colleagues (Sattlecker et al. 2014). **ENA78** is a ligand for CXCR3 which is linked to the ERK1/2 pathway that is altered in AD (Xia et al. 2000).

(c) Parkinson's disease

BST1 has been related to Parkinson's disease risk variants in the European population and in Japan: 4p15/BST1 (rs4698412, combined P = 1.8 × 10−6). Mental symptoms of Parkinson's include cognitive impairment and eventually dementia resulting from a loss of dopaminergic neurons in the substantia nigra (Myers et al. 2011).

Two of the proteins (**TFPI** and **ENTPD3**) have been suggested to be associated with cognitive decline by colleagues using the same cohort (Sattlecker et al. 2014).

4.5.3 A panel of AD cognitive decline-associated proteins and their implications in biological processes or diseases

The panel of proteins was involved in immune pathways such as JAK-STAT, cytokine-

cytokine interaction, and also in cell-cycle (PI3KAkt), prolactin, cancer and viral pathways. The main interest in connection with cognitive-decline associated proteins is the relation to immune function, cancer and viral pathways. In particular, in the aged population, cognitive decline can occur subsequent to the presentation of an insult to the immune system which can be a surgery, viral infection, or worse, cancer-related (Terrando et al 2011).

5 Model overview

The table gives a brief overview of all results obtained with WEKA software. It shows that the validation of the studies is weak to moderate yet (based on the correlation coefficient), two of them could not validate (Table 45). In addition, there is insufficient power of the validation sets (0-9%). Further work needs to be done, including larger sample sets, better patient selection (more subjects with moderate to high WMH volumes, subjects with stronger decline, for instance more than 2 annual points on the MMSE.

Strength of correlations for all models				
	Training – correlation		Validation - correlation	
WMH/mRNA (AD, MCI, CTL)	N=138, ***R*=0.66**, R^2=0.43, π = 0.99	Moderate	N= 46, ***R*=0.00**, R^2=-0.18, π = 0.00	Zero
WMH/mRNA (AD)	N=53, ***R*=0.87**, R^2=0.75, π = 0.98	Strong	N=21, ***R*=0.33**, R^2=0.11, π = 0.08	Moderate
WMH/proteins (AD, MCI, CTL)	N=136, ***R*=0.80**, R^2=0.64, π = 0.99	Strong	N=46, ***R*=0.30**, R^2=0.09, π = 0.11	Weak
WMH/proteins (AD)	N=59, ***R*=0.87**, R^2=0.76, π = 0.99	Strong	N=30, ***R*=0.17**, R^2=0.03, π = 0.06	Weak
CogDec/mRNA (AD)	N=46, ***R*=0.93**, R^2=0.86, π = 0.97	Strong	N=16, ***R*=0.36**, R^2=0.13, π = 0.06	Moderate
CogDec/proteins (AD)	N=46, ***R*=0.59**, R^2=0.35, π = 0.97	Moderate	N=16, ***R*=0.00**, R^2=0.00, π = 0.00	Zero

Table 45: Correlation thresholds: R=Strength of correlation; π=power; π = 0.80 standard for power adequacy

PART III: FUTURE DIRECTIONS

1 The potential of blood-based markers for predicting disease progression

There is a pressing lack of blood biomarkers in AD (Saad et al. 2011; Thambisetty et al. 2010; Humpel 2011; Gupta et al. 2013). This study can contribute to facilitate future efforts aimed to test new blood biomarkers in larger patient cohorts. In addition, the findings suggest a panel of candidate mRNA and proteins associated with brain pathology of WMH, and clinical cognitive measures of disease decline in AD, which may readily serve to facilitate decision-making when measuring AD progression. Clearly, the aim is to find easily obtainable markers and blood-based biomarkers bear an enormous potential in this respect. The identified mRNAs and proteins might serve as potential blood markers for WMH and AD progression which may be useful for patient selection for clinical trials. However, they require careful validation because peripheral blood is a complex tissue containing a huge number of proteins. Thus, it is currently difficult to use protein biomarkers in most clinical applications. However, in cancer treatment several new proteomic technologies have been developed for early detection, therapeutic targeting and, patient-tailored therapy (Bazenet & Lovestone 2012). Hopefully, a similar approach can be used in treatment of AD. It is often cost expensive to validate the proteins and the techniques have been slow in the past and plasma/serum mixtures often not uniform (can contain conterminants). Nevertheless, there are several blood-based biomarkers, potentially important for prediction of conversion to AD and AD diagnosis, recently identified (Lunnon et al. 2014). Many more blood-based biomarkers can possibly still be discovered with new, faster, quantitative methods.

The identified mRNA and protein signatures can be used to select experiments and facilitate the development and evaluation of therapeutics to treat Alzheimer's disease. The transcripts and proteins discovered during the course of this study could have the potential to be included in a novel biomarker panel for predicting AD-specific disease decline. Identifying how these mechanisms may vary in different patients could lead to a new horizon in the science of pharmacogenetics.

There is a non-negligible epigenetic impact on these signatures which can vary from cell type to cell type. Characterising the individual codes will enable better treatment, but also better patient stratification for clinical trials in the first place. Several vascular, environmental, psychological and genetic risk factors can have an impact on the AD disease progression (Lunnon et al. 2013; Kiddle et al. 2014; Hye et al. 2014; Sattlecker et al. 2014), some of these factors can be reflected in blood signatures. The discovery of these small molecules is challenging and sometimes described as “finding a needle in a haystack”, however, with sufficient target selectivity relevant biomarkers such as protein signatures can be found that correlate with drug activity.

2 New techniques

It can be speculated than novel image sequences, post-processing techniques, and/or imaging markers could unveil other factors contributing to (or associated with) AD disease decline. The discovery of these factors and the analysis of their relationship with incidence of disease in patients could give new insights into AD.

For instance, multiplex detection platforms using blood from AD patients have been postulated as an efficient tool aimed to discover new biomarkers of the disease (Imtiaz et al. 2014). Validation of biomarkers can be performed in independent cohorts and with different classifier algorithms. These tools are capable of predicting complex signatures for a disease but clearly more research is needed. In addition, those very sensitive tools can also be biased, for example, blood signatures can possibly be highly dependent on environmental factors such as diet or toxic air pollution that have also been proposed as risk factors for AD (Moulton & Yang 2012). The use of Bayesian algorithms could be advantageous in order to combine empirical evidence with newly identified blood signatures (Liu et al. 2013).

Moreover, gene expression analyses have recently measured presence and abundance of RNA species (by means of microarray or RNAseq [also called Whole Transcriptome Shotgun Sequencing, is a technology that uses the capabilities of next-generation sequencing to reveal a snapshot of RNA presence and quantity from a genome at a given moment in time]) in blood from AD patients. Expression profiling provides novel insights about gene behaviour under various conditions. Microarray technology can produce relatively reliable expression profiles. This technology can certainly assist formulating new biological hypothesis or help explain existing ones. However, careful interpretation, biological replication- and follow up experiments of expression profiling are required, and the experiments are time consuming. Global profiling of gene expression by microarray technology, comparing the expression level between normal and pathological, may not be the best approach. It has been suggested that measures of

interindividual-variation in gene expression may more effectively identify disease/trait-associated genes (Gorlov et al. 2014). Associations of newly identified WMH-related mRNAs and proteins with known AD related molecules could be investigated in future.

One of the possible disadvantages of this approach is that it might not necessarily reflect the blood proteome since many post-translational mechanisms can fine-tune expression of proteins in blood cells. New emerging techniques should therefore also be considered such as ribosome profiling which can more precisely quantify the proteins produced in cells and at the same time provide new insights in the mechanisms of protein synthesis (Ingolia et al. 2014). These powerful proteomic-based technologies could have many possible applications in target discovery and validation, drug discovery and response efficacy, disease mechanisms, patient stratification, or even clinical diagnosis.

Another aspect to consider when studying AD is that most pioneering studies usually show large disease effect sizes which are not always confirmed by subsequent experiments. This fact highlights the importance of biological validation in several model systems and also probably the need for developing better standardized testing tools and platforms. For example, publicly available databases of mRNA and protein biomarker panels are needed for replication and testing the validity of various methods and biological findings.

3 A blood test for Alzheimer's disease

An easily detectable blood-based biomarker for AD-disease progression has still not been found. Such a biomarker would be extremely useful in clinical trials for predicting short-term cognitive decline, and thus to determine more accurately the efficacy of potential treatments for Alzheimer's disease.

The findings of this thesis can bring this goal one step closer. A number of plausible biological associations have been identified. Following the discovery of these associations for assessing AD progression, the implicated panel of candidate proteins and mRNAs will need to undergo further evaluation with other independent groups of AD patients and in clinical trials (Figure 18).

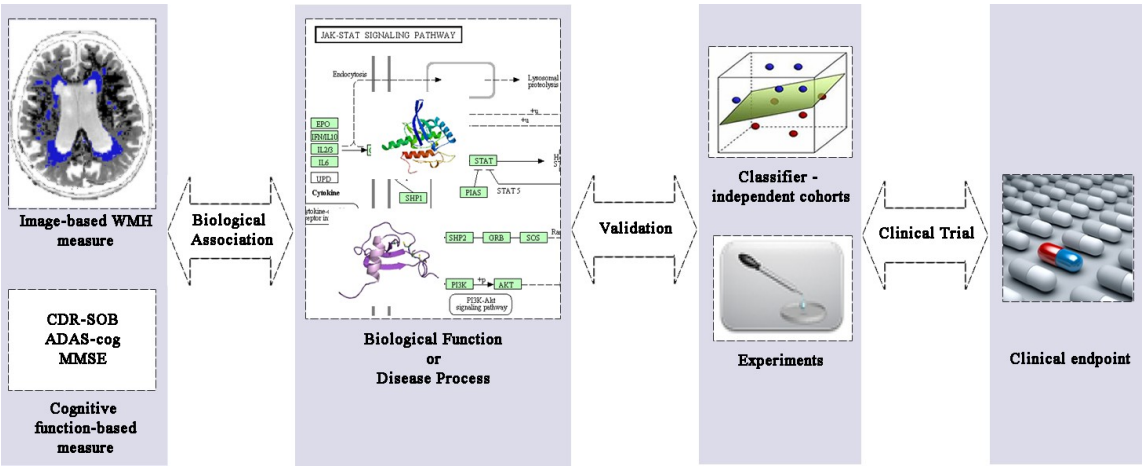

Figure 18: Three-step process for biomarker validation: The biological association establishes, by means of a classifier, the link between image-based or cognitive-based measure and the underlying molecular functions/processes. Subsequent validation can be performed by classification of the mRNA and proteins in independent cohorts and in lab experiments determining their detailed function. The validated proteins and mRNA panel identifiable with a simple blood test could qualify as endpoints in clinical trials.

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Appendices

A. Calculation of the rates of cognitive decline

CDR-SOB model of cognitive decline including WMH, atrophy, gender

```
Linear mixed-effects model fit by REML
 Data: final_mmse_5_points
       AIC      BIC    logLik
  1575.671 1622.921 -775.8355

Random effects:
 Formula: ~days | id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev      Corr
(Intercept) 1.799591820 (Intr)
days        0.003845308 0.121

 Formula: ~days | site %in% id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev      Corr
(Intercept) 1.799839460 (Intr)
days        0.003845465 0.121
Residual    1.114349012

Fixed effects: cdr ~ days + WMH + atrophy + sex
               Value Std.Error  DF   t-value p-value
(Intercept)  38.76920  7.385170 299  5.249601  0.0000
days          0.00435  0.000750 299  5.798993  0.0000
WMH           0.00011  0.000044  80  2.490906  0.0148
atrophy     -39.46981  8.918488  80 -4.425617  0.0000
sexMale      -2.11156  0.710057  80 -2.973788  0.0039
 Correlation:
        (Intr) days   WMH    atrphy
days    -0.004
WMH     -0.019 -0.004
atrophy -0.999  0.002 -0.004
sexMale -0.394 -0.002 -0.301  0.377

Standardized Within-Group Residuals:
         Min           Q1          Med           Q3          Max
-3.507413362 -0.400816759 -0.005708781  0.382728121  3.934816288

Number of Observations: 384
Number of Groups:
          id1 site %in% id1
           84            84
```

Table 46: Protocol of linear mixed model for the calculation of the rate of cognitive decline using CDR-SOB assessment scores and WMH, atrophy, and gender as covariates.

CDR-SOB model of cognitive decline including WMH, atrophy, gender, education, age of onset, apoe, disease duration

```
Linear mixed-effects model fit by REML
 Data: raw_data_5_points
       AIC      BIC    logLik
  1598.614 1661.445 -783.3072

Random effects:
 Formula: ~days | id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev      Corr
(Intercept) 1.808797407 (Intr)
days        0.003846545 0.113

 Formula: ~days | site %in% id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev      Corr
(Intercept) 1.809328411 (Intr)
days        0.003846825 0.113
Residual    1.114204094

Fixed effects: cdr ~ days + education + apoe + sex + atrophy + WMH +
onset +      duration_base
                 Value Std.Error  DF   t-value p-value
(Intercept)    46.56290 10.234640 299  4.549540  0.0000
days            0.00435  0.000750 299  5.797735  0.0000
education      -0.10266  0.072887  76 -1.408492  0.1631
apoe           -0.00964  0.069244  76 -0.139266  0.8896
sexMale        -1.93298  0.730613  76 -2.645700  0.0099
atrophy       -42.60522  9.776859  76 -4.357761  0.0000
WMH             0.00011  0.000047  76  2.293003  0.0246
onset          -0.05920  0.054395  76 -1.088380  0.2799
duration_base   0.00408  0.010710  76  0.380655  0.7045
 Correlation:
              (Intr) days   eductn apoe   sexMal atrphy WMH    onset
days          -0.001
education     -0.243  0.000
apoe          -0.196  0.000  0.009
sexMale       -0.163 -0.001 -0.070 -0.164
atrophy       -0.873  0.000  0.167 -0.137  0.319
WMH            0.127 -0.003  0.140 -0.151 -0.240 -0.039
onset         -0.635 -0.003  0.125  0.206 -0.162  0.252 -0.218
duration_base -0.369 -0.002  0.012 -0.025 -0.015  0.284 -0.166  0.301

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.51898401 -0.40229829 -0.01051205  0.37960626  3.92883022

Number of Observations: 384
Number of Groups:
          id1 site %in% id1
           84            84
```

Table 47: Protocol of linear mixed model for the calculation of the rate of cognitive decline using CDR-SOB assessment scores and WMH, atrophy, gender, education, disease onset, disease duration, and apoe, as covariates.

MMSE model of cognitive decline including including WMH, atrophy, gender

```
Linear mixed-effects model fit by REML
 Data: final_mmse_5_points
       AIC      BIC    logLik
  1966.415 2013.697 -971.2073

Random effects:
 Formula: ~days | id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev      Corr
(Intercept) 2.929846314 (Intr)
days        0.004597678 0.17

 Formula: ~days | site %in% id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev      Corr
(Intercept) 2.927742571 (Intr)
days        0.004598807 0.17
Residual    1.975110168

Fixed effects: mmse ~ days + WMH + atrophy + sex
                Value Std.Error  DF   t-value p-value
(Intercept) -3.801763 12.101304 300 -0.314161  0.7536
days        -0.003699  0.001066 300 -3.471109  0.0006
WMH         -0.000173  0.000072  80 -2.396644  0.0189
atrophy     30.987866 14.613679  80  2.120470  0.0371
sexMale      2.432103  1.163068  80  2.091110  0.0397
 Correlation:
        (Intr) days   WMH    atrphy
days    -0.003
WMH     -0.019 -0.003
atrophy -0.999 -0.001 -0.004
sexMale -0.395 -0.003 -0.300  0.378

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.66162938 -0.50517096  0.03818177  0.55234867  2.60605259

Number of Observations: 385
Number of Groups:
          id1 site %in% id1
           84            84
```

Table 48: Protocol of linear mixed model for the calculation of the rate of cognitive decline using MMSE assessment scores and WMH, atrophy, and gender as covariates.

MMSE model of cognitive decline including including WMH, atrophy, gender, education, age of onset, apoe, disease duration

```
Linear mixed-effects model fit by REML
 Data: raw_data_5_points
       AIC     BIC    logLik
  1979.677 2042.55 -973.8383

Random effects:
 Formula: ~days | id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev      Corr
(Intercept) 2.802085482 (Intr)
days        0.004640754 0.282

 Formula: ~days | site %in% id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev      Corr
(Intercept) 2.800850605 (Intr)
days        0.004634611 0.282
Residual    1.973124029

Fixed effects: mmse ~ days + education + apoe + sex + atrophy + WMH +
onset +      duration_base
                 Value Std.Error  DF   t-value p-value
(Intercept)   -18.32560 16.132594 300 -1.135937  0.2569
days           -0.00374  0.001069 300 -3.496156  0.0005
education       0.34850  0.114868  76  3.033895  0.0033
apoe            0.02728  0.109033  76  0.250158  0.8031
sexMale         2.05394  1.151418  76  1.783837  0.0784
atrophy        39.70781 15.407461  76  2.577181  0.0119
WMH            -0.00014  0.000074  76 -1.954058  0.0544
onset           0.04036  0.085742  76  0.470672  0.6392
duration_base   0.01366  0.016881  76  0.809233  0.4209
 Correlation:
              (Intr) days   eductn apoe   sexMal atrphy WMH    onset
days          -0.001
education     -0.243  0.001
apoe          -0.196  0.002  0.009
sexMale       -0.162 -0.002 -0.070 -0.163
atrophy       -0.873  0.002  0.167 -0.136  0.319
WMH            0.127 -0.004  0.140 -0.151 -0.240 -0.039
onset         -0.636 -0.004  0.125  0.207 -0.163  0.253 -0.218
duration_base -0.368 -0.002  0.012 -0.025 -0.015  0.284 -0.166  0.301

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.70305707 -0.50868256  0.03809261  0.55789575  2.58039213

Number of Observations: 385
Number of Groups:
          id1 site %in% id1
           84            84
```

Table 49: Protocol of linear mixed model for the calculation of the rate of cognitive decline using MMSE assessment scores and WMH, atrophy, gender, education, disease onset, disease duration and APOE genotype as covariates.

ADAS-cog model of cognitive decline including including WMH, atrophy, gender

```
Linear mixed-effects model fit by REML
 Data: final_mmse_5_points
       AIC      BIC    logLik
  2483.893 -1206.321

Random effects:
 Formula: ~days | id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev    Corr
(Intercept) 6.4143455 (Intr)
days        0.0109157 0.173

 Formula: ~days | site %in% id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev     Corr
(Intercept) 6.41062259 (Intr)
days        0.01091673 0.173
Residual    3.40142932

Fixed effects: adas ~ days + sex + atrophy + WMH
                Value Std.Error  DF   t-value p-value
(Intercept)  60.21001 25.983283 299  2.317260  0.0212
days          0.00529  0.002189 299  2.414890  0.0163
sexMale      -4.85282  2.498470  80 -1.942318  0.0556
atrophy     -45.69813 31.378791  80 -1.456338  0.1492
WMH           0.00033  0.000155  80  2.114972  0.0375
 Correlation:
        (Intr) days   sexMal atrphy
days    -0.005
sexMale -0.394 -0.001
atrophy -0.999  0.004  0.377
WMH     -0.019 -0.004 -0.300 -0.004

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.55022713 -0.58105258  0.04297236  0.52667560  2.34827157

Number of Observations: 384
Number of Groups:
          id1 site %in% id1
           84            84
```

Table 50: Protocol of linear mixed model: rate of cognitive decline based on ADAS-cog assessment with covariates WMH, atrophy, and gender

ADAS-cog model of cognitive decline including including WMH, atrophy, gender, education, age of onset, apoe, disease duration

```
Linear mixed-effects model fit by REML
 Data: raw_data_5_points
       AIC      BIC    logLik
  2443.386 2506.217 -1205.693

Random effects:
 Formula: ~days | id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev     Corr
(Intercept) 6.16184454 (Intr)
days        0.01093556 0.2

 Formula: ~days | site %in% id1
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev     Corr
(Intercept) 6.16078497 (Intr)
days        0.01093605 0.2
Residual    3.40043494

Fixed effects: adas ~ days + education + apoe + sex + atrophy + WMH +
onset +      duration_base
                 Value Std.Error  DF    t-value p-value
(Intercept)   100.33152  34.56727 299  2.9025007  0.0040
days            0.00530   0.00219 299  2.4209372  0.0161
education      -0.75087   0.24618  76 -3.0500639  0.0031
apoe           -0.12545   0.23372  76 -0.5367684  0.5930
sexMale        -3.97511   2.46803  76 -1.6106388  0.1114
atrophy       -66.54302  33.01758  76 -2.0153816  0.0474
WMH             0.00029   0.00016  76  1.8631427  0.0663
onset          -0.16012   0.18375  76 -0.8713831  0.3863
duration_base  -0.02474   0.03619  76 -0.6838194  0.4962
 Correlation:
              (Intr) days   eductn apoe   sexMal atrphy WMH    onset
days          -0.003
education     -0.243  0.001
apoe          -0.196  0.001  0.009
sexMale       -0.162 -0.001 -0.070 -0.163
atrophy       -0.873  0.004  0.167 -0.136  0.319
WMH            0.128 -0.004  0.140 -0.151 -0.240 -0.039
onset         -0.635 -0.001  0.125  0.207 -0.163  0.252 -0.218
duration_base -0.369 -0.001  0.012 -0.025 -0.015  0.285 -0.167  0.301

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.57664622 -0.58138819  0.04073876  0.52885561  2.38275132

Number of Observations: 384
Number of Groups:
          id1 site %in% id1
           84            84
```

Table 51: Protocol of linear mixed model: rate of cognitive decline based on ADAS-cog assessment with covariates WMH, atrophy, gender, education, disease onset, duration since disease onset, and APOE genotype as covariates.

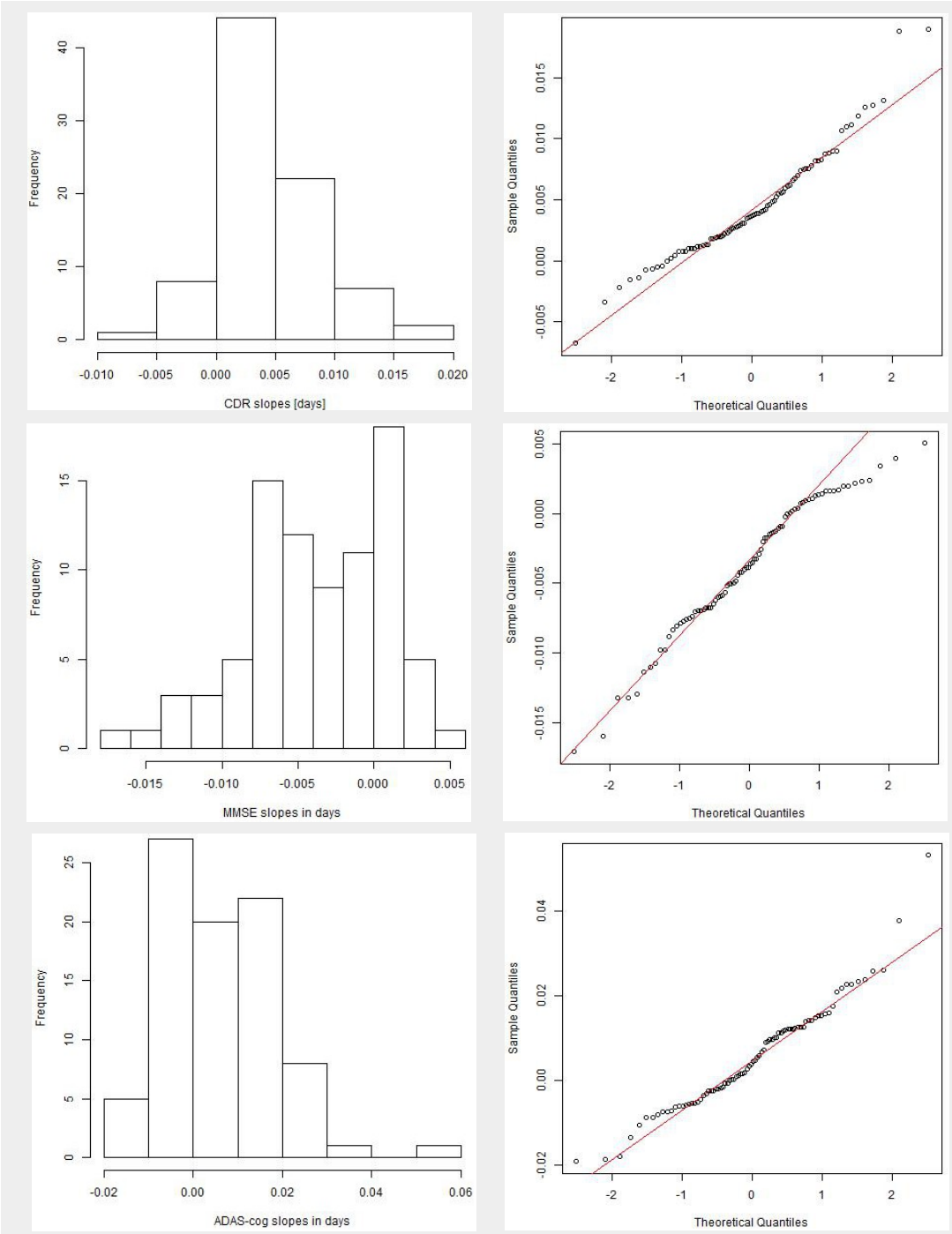

Figure 19: 1st row: rate of cognitive decline baseline model: histogram of the CDR-SOB cognitive decline slopes (left), Quantile-Quantile- plot of the CDR-SOB cognitive decline slopes (right); 2nd row rate of cognitive decline baseline model:histogram of the MMSE cognitive decline slopes (left),QQ- plot of the MMSE cognitive decline slopes (right); 3rd row: rate of cognitive decline baseline model:histogram of the ADAS-cog cognitive decline slopes (left),QQ- plot of the ADAS-cog cognitive decline slopes (right)

Power calculation of the CDR-SOB model – rate of cognitive decline
http://cran.r-project.org/web/packages/longpower/vignettes/longpower.pdf (Donohue et al. 2013)

```
library(longpower)

liu.liang.linear.power
function (N = NULL, delta = NULL, u = NULL, v = NULL, sigma2 = 1,
        R = NULL, R.list = NULL, sig.level = 0.05, power = NULL,
        Pi = rep(1/length(u), length(u)), alternative = c("two.sided", "one.sided"))
 {
  if (sum(sapply(list(N, delta, sigma2, power, sig.level),
              is.null)) != 1)
    stop("exactly one of 'N', 'sigma2', 'delta', 'power', and 'sig.level' must be NULL")
  if (!is.null(sig.level) && !is.numeric(sig.level) || any(0 >
                                          sig.level | sig.level > 1))
    stop("'sig.level' must be numeric in [0, 1]")
  alternative <- match.arg(alternative)
  if (sum(c(!is.null(R), !is.null(R.list))) != 1)
    stop("Exactly one of R or R.list must be specified.")
  if (sum(Pi) != 1)
    stop("Pi must sum to 1.")
  if (!is.null(R)) {
    R.list <- lapply(1:length(u), function(i) R)
  }
  Rinv <- lapply(1:length(R.list), function(i) {
    R <- R.list[[i]]
    if (is.null(dim(R)) & length(R) == 1 & length(u[[i]]) >
        1) {
      R <- matrix(R, length(u[[i]]), length(u[[i]])) +
        diag(1 - R, length(u[[i]]))
    }
    else if (is.null(dim(R)) & length(R) == 1 & length(u[[i]]) ==
            1) {
      R <- matrix(R, length(u[[i]]), length(u[[i]]))
    }
    return(solve(R))
  })
  n.body <- quote({
    Ipl <- 0
    for (i in 1:length(u)) Ipl <- Ipl + Pi[i] * t(u[[i]]) %*%
      Rinv[[i]] %*% v[[i]]
    Ipl <- Ipl/sigma2
    Ill <- 0
    for (i in 1:length(u)) Ill <- Ill + Pi[i] * t(v[[i]]) %*%
      Rinv[[i]] %*% v[[i]]
    Illinv <- solve(Ill/sigma2)
    Sigma1 <- 0
    for (i in 1:length(u)) Sigma1 <- Sigma1 + Pi[i] * (t(u[[i]]) -
                                        Ipl %*% Illinv %*% t(v[[i]])) %*% Rinv[[i]] %*% (u[[i]] -
                                                                v[[i]] %*% Illinv %*% t(Ipl))
    Sigma1 <- Sigma1/sigma2
    n1 <- (qnorm(1 - ifelse(alternative == "two.sided", sig.level/2,
                        sig.level)) + qnorm(power))^2/(delta %*% Sigma1 %*%
                                            delta)[1, 1]
    n <- n1/Pi[1] * Pi
    sum(n)
  })
  if (is.null(sig.level))
    sig.level <- uniroot(function(sig.level) eval(n.body) -
                         N, c(1e-10, 1 - 1e-10))$root
```

```
  else if (is.null(power))
    power <- uniroot(function(power) eval(n.body) - N, c(0.001,
                                        31 - 1e-10))$root
  else if (is.null(delta))
    delta <- uniroot(function(delta) eval(n.body) - N, c(1e-10,
                                        1e+05))$root
  else if (is.null(sigma2))
    sigma2 <- uniroot(function(sigma2) eval(n.body) - N,
                  c(1e-10, 1e+05))$root
  N <- eval(n.body)
  METHOD <- "Longitudinal linear model power calculation (Liu & Liang, 1997)"
  structure(list(N = N, n = N * Pi, delta = delta, sigma2 = sigma2,
                 sig.level = sig.level, power = power, alternative = alternative,
                 R = R, note = "N is total sample size and n is sample size in each group.",
                 method = METHOD), class = "power.longtest")
 }
#<environment: namespace:longpower>

# var of random intercept
sig2.i = 3.23853
# var of random slope
sig2.s = 0.000014786332
# residual var
sig2.e = 1.2417736938
# covariance of slope and intercep
cov.s.i <- 0.8*sqrt(sig2.i)*sqrt(sig2.s)
cov.t <- function(t1, t2, sig2.i, sig2.s, cov.s.i){
 sig2.i + t1*t2*sig2.s + (t1+t2)*cov.s.i
}
t = seq(0,1.5,0.25)
n = length(t)
R = outer(t, t, function(x,y){cov.t(x,y, sig2.i, sig2.s, cov.s.i)})
R = R + diag(sig2.e, n, n)
u = list(u1 = t, u2 = rep(0,n))
v = list(v1 = cbind(1,1,rep(0,n)),
       v2 = cbind(1,0,t))
```

```
> liu.liang.linear.power(d=0.5, u=u, v=v, R=R, sig.level=0.05,
power=0.80)

     Longitudinal linear model power calculation (Liu & Liang, 1997)

               N = 89.11301
               n = 44.55651, 44.55651
           delta = 0.5
          sigma2 = 1
       sig.level = 0.05
           power = 0.8
     alternative = two.sided

 NOTE: N is total sample size and n is sample size in each group.

 R:
          [,1]     [,2]     [,3]     [,4]     [,5]     [,6]     [,7]
[1,] 4.480304 3.239914 3.241298 3.242682 3.244066 3.245450 3.246834
```

```
[2,] 3.239914 4.483073 3.242684 3.244069 3.245454 3.246839 3.248224
[3,] 3.241298 3.242684 4.485843 3.245456 3.246841 3.248227 3.249613
[4,] 3.242682 3.244069 3.245456 4.488616 3.248229 3.249616 3.251003
[5,] 3.244066 3.245454 3.246841 3.248229 4.491390 3.251004 3.252392
[6,] 3.245450 3.246839 3.248227 3.249616 3.251004 4.494167 3.253782
[7,] 3.246834 3.248224 3.249613 3.251003 3.252392 3.253782 4.496945
```

Table 52: Power calculation of the CDR-SOB model – rate of cognitive decline

B. Listing of identified mRNA, proteins and regression protocol

(1a) mRNA transcripts associated with WMH identified with features selection

	Full name	Chromo-somal location	Gene Name [Alias] Gene ID	Description
1	aminolevulinate, delta-, synthase 2	Xp11.21	ALAS2 212	apoptotic nuclear changes, epidermis development, keratinization, mitochondrion organization, negative regulation of inflammatory response, positive regulation of I-kappaB kinase/NF-kappaB signaling, protein homooligomerization, protein linear polyubiquitination, regulation of CD40 signaling pathway, regulation of tumor necrosis factor-mediated signaling pathway
2	ADP-ribosylation factor GTPase activating protein 1	20q13.33	ARFGAP1 55738	GTPase-activating protein (GAP) for the ADP ribosylation factor 1 (ARF1). Involved in membrane trafficking and /or vesicle transport. Promotes hydrolysis of the ARF1-bound GTP and thus, is required for the dissociation of coat proteins from Golgi-derived membranes and vesicles, a prerequisite for vesicle's fusion with target compartment. Probably regulates ARF1-mediated transport via its interaction with the KDELR proteins and RNP24. Overexpression induces the redistribution of the entire Golgi complex to the endoplasmic reticulum.
3	mitochondrial calcium uniporter regulator 1	6p23	CCDC90A [MCUR1] 63933	calcium ion import, mitochondrial calcium ion transport, positive regulation of mitochondrial calcium ion

	Full name	Chromo-somal location	Gene Name [Alias] Gene ID	Description
				concentration
4	cAMP responsive element binding protein 5	7p15	CREB5 9586	binds to the cAMP response element and activates transcription
5	eomesodermin	3p24.1	EOMES	Functions as a transcriptional activator playing a crucial role during development. Functions in trophoblast differentiation and later in gastrulation, regulating both mesoderm delamination and endoderm specification. Plays a role in brain development being required for the specification and the proliferation of the intermediate progenitor cells and their progeny in the cerebral cortex. Also involved in the differentiation of CD8+ T-cells during immune response regulating the expression of lytic effector genes
6	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	11q13	FAU 2197	This gene is the cellular homolog of the fox sequence in the Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV). It encodes a fusion protein consisting of the ubiquitin-like protein fubi at the N terminus and ribosomal protein S30 at the C terminus. It has been proposed that the fusion protein is post-translationally processed to generate free fubi and free ribosomal protein S30. Fubi is a member of the ubiquitin family, and ribosomal protein S30 belongs to the S30E family of ribosomal proteins. Whereas the function of fubi is currently unknown, ribosomal protein S30 is a component of the 40S subunit of the cytoplasmic ribosome. Pseudogenes derived from this gene are present in the genome. Similar to ribosomal protein S30, ribosomal proteins S27a and L40 are synthesized as fusion proteins with ubiquitin.
7	F-box and WD repeat domain containing 4	10q24	FBXW4 6468	Probably recognizes and binds to some phosphorylated proteins and promotes their ubiquitination and degradation. Likely to be involved in key signaling pathways crucial for normal limb development. May participate in Wnt signaling
8	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	19q13.4	LILRA5 353514	May plays a role in triggering innate immune responses. Seems not play a role for any class I MHC antigens recognition

	Full name	Chromo-somal location	Gene Name [Alias] Gene ID	Description
9	non-SMC condensin I complex, subunit D2	12p13.31	NCAPD2 9918	egulatory subunit of the condensin complex, a complex required for conversion of interphase chromatin into mitotic-like condense chromosomes. The condensin complex probably introduces positive supercoils into relaxed DNA in the presence of type I topoisomerases and converts nicked DNA into positive knotted forms in the presence of type II topoisomerases. May target the condensin complex to DNA via its C-terminal domain
10	paroxysmal nonkinesigenic dyskinesia	2q35	PNKD 25953	Probable hydrolase that plays an aggravative role in the development of cardiac hypertrophy via activation of the NF-kappa- B signaling pathway
11	Ras-related GTP binding C	1p34	RRAGC 64121	Has guanine nucleotide-binding activity but weak intrinsic GTPase activity
12	SHANK-associated RH domain interacting protein	8q24.3	SHARPIN 106025	May have a role in normal immune development and control of inflammation
13	ZNRD1 antisense RNA 1	6p21.33	ZNRD1A [C6orf1] 80862	RNA, long non-coding

Table 53: Description of WMH-associated mRNAs

(1b) Summary of test statistics from feature selection mRNA *versus* WMH

```
1. ALAS

Residuals:
   Min     1Q Median     3Q    Max
 -8591  -4560  -2870   2305  41009

Coefficients:
              Estimate Std. Error t value Pr(>|t|)
(Intercept)       5282        664   7.954 6.24e-13 ***
data$V3          12576       6356   1.979   0.0499 *

Residual standard error: 7797 on 136 degrees of freedom
Multiple R-squared:  0.02798,   Adjusted R-squared:  0.02083
F-statistic: 3.915 on 1 and 136 DF,  p-value: 0.04989
============================================================
2. ARFGAP1

Residuals:
   Min     1Q Median     3Q    Max
 -7734  -4898  -2336   2004  38821
```

```
Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5096.6      652.6   7.810 1.38e-12 ***
data$V2      -12452.0     4045.6  -3.078  0.00252 **

Residual standard error: 7646 on 136 degrees of freedom
Multiple R-squared:  0.06512,   Adjusted R-squared:  0.05825
F-statistic: 9.474 on 1 and 136 DF,  p-value: 0.002521
=================================================================
3. CC2D1B

Residuals:
   Min     1Q Median     3Q    Max
 -8591  -4560  -2870   2305  41009

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5282        664   7.954 6.24e-13 ***
data$V3       12576       6356   1.979   0.0499 *


Residual standard error: 7797 on 136 degrees of freedom
Multiple R-squared:  0.02798,   Adjusted R-squared:  0.02083
F-statistic: 3.915 on 1 and 136 DF,  p-value: 0.04989
=================================================================
4. CCDC90A

Residuals:
   Min     1Q Median     3Q    Max
 -9033  -4540  -2844   2664  41030

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5344.9      655.6   8.152 2.09e-13 ***
data$V4       14586.4     5208.1   2.801  0.00584 **


Residual standard error: 7690 on 136 degrees of freedom
Multiple R-squared:  0.05453,   Adjusted R-squared:  0.04758
F-statistic: 7.844 on 1 and 136 DF,  p-value: 0.005842
=================================================================
5. CREB5

Residuals:
   Min     1Q Median     3Q    Max
 -7734  -4642  -2491   2054  40167

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5119.7      664.4   7.705 2.45e-12 ***
data$V5       11850.8     5517.0   2.148   0.0335 *


Residual standard error: 7777 on 136 degrees of freedom
Multiple R-squared:  0.03281,   Adjusted R-squared:  0.0257
F-statistic: 4.614 on 1 and 136 DF,  p-value: 0.03348
=================================================================
6. EOMES

Residuals:
   Min     1Q Median     3Q    Max
 -9909  -4256  -1985   1970  37236

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5398.2      648.3   8.327  7.9e-14 ***
```

```
      data$V6   9162.8     2712.9   3.378 0.000954 ***


Residual standard error: 7596 on 136 degrees of freedom
Multiple R-squared:  0.07739,   Adjusted R-squared:  0.07061
F-statistic: 11.41 on 1 and 136 DF,  p-value: 0.0009541
============================================================
7. FAU

Residuals:
   Min     1Q Median     3Q    Max
 -9088  -4860  -2578   2297  40098

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)          5326.0      656.2   8.116 2.55e-13 ***
      data$V7  16484.6     6039.8   2.729  0.00719 **


Residual standard error: 7700 on 136 degrees of freedom
Multiple R-squared:  0.05193,   Adjusted R-squared:  0.04496
F-statistic: 7.449 on 1 and 136 DF,  p-value: 0.007185
============================================================
8. FBXW4

Residuals:
   Min     1Q Median     3Q    Max
 -9666  -4363  -2052   2297  38849

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)          5223.9      641.9   8.138 2.26e-13 ***
     data$V8 -16574.0     4495.7  -3.687 0.000327 ***


Residual standard error: 7540 on 136 degrees of freedom
Multiple R-squared:  0.09086,   Adjusted R-squared:  0.08417
F-statistic: 13.59 on 1 and 136 DF,  p-value: 0.0003273
============================================================
9. HS.127310

Residuals:
   Min     1Q Median     3Q    Max
 -8932  -4581  -2797   2448  39092

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)          5230.9      656.3   7.970 5.72e-13 ***
      data$V9 -22635.1     8500.4  -2.663  0.00868 **


Residual standard error: 7710 on 136 degrees of freedom
Multiple R-squared:  0.04955,   Adjusted R-squared:  0.04257
F-statistic: 7.091 on 1 and 136 DF,  p-value: 0.008684
============================================================
10. LILRA5

Residuals:
   Min     1Q Median     3Q    Max
-10897  -4667  -2461   2481  35889

Coefficients:
              Estimate Std. Error t value Pr(>|t|)
(Intercept)           4997.9      653.7   7.646 3.38e-12 ***
       data$V10  -2622.1      820.8  -3.194  0.00174 **
```

```
Residual standard error: 7627 on 136 degrees of freedom
Multiple R-squared:  0.0698,    Adjusted R-squared:  0.06296
F-statistic:  10.2 on 1 and 136 DF,  p-value: 0.001741
================================================================
11. NAG18

Residuals:
   Min     1Q Median     3Q    Max
 -9692  -4514  -2951   1442  40159

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5179.9      656.7   7.888 8.98e-13 ***
data$V11  17742.3     6655.2   2.666  0.00861 **


Residual standard error: 7709 on 136 degrees of freedom
Multiple R-squared:  0.04966,   Adjusted R-squared:  0.04268
F-statistic: 7.107 on 1 and 136 DF,  p-value: 0.008608
================================================================
12. NCAPD2

Residuals:
   Min     1Q Median     3Q    Max
-10994  -4501  -2370   2022  40078

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5098.8      649.1   7.855 1.08e-12 ***
data$V12  11389.3     3443.8   3.307  0.00121 **


Residual standard error: 7608 on 136 degrees of freedom
Multiple R-squared:  0.07444,   Adjusted R-squared:  0.06763
F-statistic: 10.94 on 1 and 136 DF,  p-value: 0.001206
================================================================
13. NRBP2

Residuals:
   Min     1Q Median     3Q    Max
 -9394  -4840  -2316   2190  40441

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5122.9      660.9   7.751  1.9e-12 ***
data$V13  14507.6     5990.7   2.422   0.0168 *


Residual standard error: 7743 on 136 degrees of freedom
Multiple R-squared:  0.04134,   Adjusted R-squared:  0.03429
F-statistic: 5.865 on 1 and 136 DF,  p-value: 0.01677
================================================================
14. PNKD

Residuals:
   Min     1Q Median     3Q    Max
-11900  -4185  -2136   1362  42811

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    5318.2      640.7   8.301 9.13e-14 ***
data$V14  21596.5     5709.6   3.783 0.000232 ***


Residual standard error: 7523 on 136 degrees of freedom
Multiple R-squared:  0.09519,   Adjusted R-squared:  0.08853
F-statistic: 14.31 on 1 and 136 DF,  p-value: 0.0002318
```

```
==========================================================================
15. RRAGC

Residuals:
   Min     1Q Median     3Q    Max
 -9024  -4517  -2209   1759  40666

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       5256.6      654.1   8.036 3.97e-13 ***
      data$V15  11927.2     4199.3   2.840   0.0052 **


Residual standard error: 7684 on 136 degrees of freedom
Multiple R-squared:  0.056,       Adjusted R-squared:  0.04905
F-statistic: 8.067 on 1 and 136 DF,  p-value: 0.005201
==========================================================================
16. SHARPIN

Residuals:
   Min     1Q Median     3Q    Max
 -7668  -4485  -2604   1680  42006

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       5178        658   7.869 9.97e-13 ***
       data$V16   -11759       4594  -2.560   0.0116 *


Residual standard error: 7724 on 136 degrees of freedom
Multiple R-squared:  0.04596,   Adjusted R-squared:  0.03895
F-statistic: 6.552 on 1 and 136 DF,  p-value: 0.01157
==========================================================================
17. ZNRD1A

Residuals:
   Min     1Q Median     3Q    Max
 -8213  -4811  -2467   2274  39743

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       5166.4      660.7   7.819 1.31e-12 ***
       data$V17  13270.9     5656.0   2.346   0.0204 *


Residual standard error: 7753 on 136 degrees of freedom
Multiple R-squared:  0.03891,   Adjusted R-squared:  0.03184
F-statistic: 5.505 on 1 and 136 DF,  p-value: 0.0204

============================================================

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Table 54: Test-statistic from selected features: mRNA versus WMH

(1c) Regression - WMH volume versus mRNA expression

In AD MCI & controls
=== Run information === Scheme:weka.classifiers.meta.CVParameterSelection -P "C 0.0 1.0 1.0" -P "R -5.99999999 5.0E-4 5.00000001" -X 10 -S 1 -W

```
weka.classifiers.functions.LinearRegression -- -S 0 -R 1.0E-8
Relation:     wml.rna_status-weka.filters.unsupervised.attribute.Reorder-R2,..-6282
Instances:    138
Attributes:   17
              ALAS2
              ARFGAP1
              CC2D1B
              CCDC90A
              CREB5
              EOMES
              FAU
              FBXW4
              HS.127310
              LILRA5
              NAG18
              NCAPD2
              NRBP2
              PNKD
              RRAGC
              SHARPIN
              WMH
Test mode:evaluate on training data

=== Classifier model (full training set) ===

Cross-validated Parameter selection.
Classifier: weka.classifiers.functions.LinearRegression
Cross-validation Parameter: '-C' ranged from 0.0 to 1.0 with 1.0 steps
Cross-validation Parameter: '-R' ranged from -5.99999999 to 5.0E-4 with 5.00000001 steps
Classifier Options: -C 0 -R 4.999849987497029E-4 -S 0


Linear Regression Model

WMH =

   8028.4474 * ALAS2 +
  -8191.0329 * ARFGAP1 +
   7635.5274 * CCDC90A +
   7693.6158 * CREB5 +
   5374.5716 * EOMES +
  10756.512  * FAU +
  -7732.4478 * FBXW4 +
  -1850.664  * LILRA5 +
   6559.3473 * NCAPD2 +
  10037.305  * PNKD +
   7043.0868 * RRAGC +
  -5684.4099 * SHARPIN +
   4896.4628

Time taken to build model: 0.13 seconds

=== Evaluation on training set ===
=== Summary ===

Correlation coefficient                  0.6551
Mean absolute error                   4197.2872
Root mean squared error               5931.9342
Relative absolute error                 77.1988 %
Root relative squared error             75.5584 %
Total Number of Instances              138

=== Cross-validation ===
=== Summary ===

Correlation coefficient                  0.39
Mean absolute error                   5436.5803
```

```
Root mean squared error              7535.5722
Relative absolute error             99.2728 %
Root relative squared error          95.4369 %
Total Number of Instances            138


=== Re-evaluation on test set ===

User supplied test set
Relation:     wml.rna_status-weka.filters.unsupervised.attribute.Reorder-R2,..,-6282
Instances:     unknown (yet). Reading incrementally
Attributes:   17

=== Summary ===

Correlation coefficient               -0.1754
Mean absolute error                8777.6568
Root mean squared error           13373.1989
Total Number of Instances            46
```

Table 55: WMH versus mRNA WEKA protocol in AD|MCI|controls

In AD

```
=== Run information ===

Scheme:weka.classifiers.meta.CVParameterSelection -P "C 0.0 1.0 1.0" -P "R -5.99999999 5.0E-4 5.00000001" -X 10 -S 1 -W
weka.classifiers.functions.LinearRegression -- -S 0 -R 1.0E-8
Relation:     wml.rna_status-weka.filters.unsupervised.attribute.Reorder-R2,...-6282
Instances:    53
Attributes:   17
              ALAS2
              ARFGAP1
              CC2D1B
              CCDC90A
              CREB5
              EOMES
              FAU
              FBXW4
              HS.127310
              LILRA5
              NAG18
              NCAPD2
              NRBP2
              PNKD
              RRAGC
              SHARPIN
              WMH
Test mode:evaluate on training data

=== Classifier model (full training set) ===

Cross-validated Parameter selection.
Classifier: weka.classifiers.functions.LinearRegression
Cross-validation Parameter: '-C' ranged from 0.0 to 1.0 with 1.0 steps
Cross-validation Parameter: '-R' ranged from -5.99999999 to 5.0E-4 with 5.00000001 steps
Classifier Options: -C 0 -R -1.4996250087509377 -S 0
Linear Regression Model

WMH =

  14288.5648 * ALAS2 +
 -11005.0273 * ARFGAP1 +
  13310.0056 * CCDC90A +
```

```
  6658.9233 * EOMES +
 15332.6058 * FAU +
 19844.9624 * HS.127310 +
 -1260.5952 * LILRA5 +
 17393.7616 * NAG18 +
 11779.5723 * NCAPD2 +
 16070.3286 * PNKD +
 -9128.7056 * SHARPIN +
  4500.5989

Time taken to build model: 0.15 seconds

=== Evaluation on training set ===
=== Summary ===

Correlation coefficient                  0.8666
Mean absolute error                   3032.5134
Root mean squared error               3994.3008
Relative absolute error                 57.5639 %
Root relative squared error             49.9262 %
Total Number of Instances               53


=== Cross-validation ===
=== Summary ===

Correlation coefficient                  0.5062
Mean absolute error                   5696.7188
Root mean squared error               7712.7217
Relative absolute error                107.0533 %
Root relative squared error             95.3073 %
Total Number of Instances               53


=== Re-evaluation on test set ===

User supplied test set
Relation:     wml.rna_status-weka.filters.unsupervised.attribute.Reorder-R2,..-6282
Instances:     unknown (yet). Reading incrementally
Attributes:   17

=== Summary ===

Correlation coefficient                  0.3366
Mean absolute error                   8607.5686
Root mean squared error              10737.527
Total Number of Instances               21
```

Table 56: WMH versus mRNA WEKA protocol in AD

(2a) Proteins associated with WMH identified with features selection

	Protein name	**Chromo-somal location**	**Associated Gene Name [Alias] Gene ID**	**String-db/ Uniprot description**
1	Chemokine (C-X-C motif) ligand 16 [Source:HGNC Symbol;Acc:16642]	17p13	CXCL16 58191	Chemokine (C-X-C motif) ligand 16; Acts as a scavenger receptor on macrophages, which specifically binds to OxLDL (oxidized low density lipoprotein), suggesting that it may be

	Protein name	Chromo-somal location	Associated Gene Name [Alias] Gene ID	String-db/ Uniprot description
				involved in pathophysiology such as atherogenesis (By similarity). Induces a strong chemotactic response. Induces calcium mobilization. Binds to CXCR6/Bonzo (273 aa)
2	Tumor necrosis factor (ligand) superfamily, member 12 [Source:HGNC Symbol;Acc:11927]	17p13.1	TWEAK [TNFSF12] 8742	Tumor necrosis factor (ligand) superfamily, member 12; Binds to FN14 and possibly also to TNRFSF12/APO3. Weak inducer of apoptosis in some cell types. Mediates NF-kappa-B activation. Promotes angiogenesis and the proliferation of endothelial cells. Also involved in induction of inflammatory cytokines (330 aa)
3	Thyroid hormone receptor interactor 13 [Source:HGNC Symbol;Acc:12307]	5p15	HPV E7 Type 16 TRIP13 9319	Thyroid hormone receptor interactor 13 (432 aa) E7 protein has both transforming and trans-activating activities. Disrupts the function of host retinoblastoma protein RB1/pRb, which is a key regulator of the cell cycle. Induces the disassembly of the E2F1 transcription factors from RB1, with subsequent transcriptional activation of E2F1-regulated S-phase genes. Inactivation of the ability of RB1 to arrest the cell cycle is critical for cellular transformation, uncontrolled cellular growth and proliferation induced by viral infection. Stimulation of progression from G1 to S phase allows the virus to efficiently use the cellular DNA replicating machinery to achieve viral genome replication. Interferes with histone deacetylation mediated by HDAC1 and HDAC2, leading to activation of transcription
4	Macrophage mannose receptor	10p13	MRC1 4360	MRC1 - mannose receptor, C type 1; Mediates the endocytosis of glycoproteins by macrophages. Binds both sulfated and non-sulfated polysaccharide chains. Acts as phagocytic receptor for bacteria, fungi and other pathogens
5	Growth differentiation factor 11	12q13.13	GDF 11 [BMP11] 10220	Secreted signal that acts globally to specify positional identity along the anterior/posterior axis during development. Play critical roles in patterning both mesodermal and neural tissues and in establishing the skeletal pattern (407 aa)
6	Alkaline phosphatase bone	1p36.12	ALPL 249	Alkaline phosphatase, liver/bone/kidney; This isozyme may play a role in skeletal mineralization
7	IgM	Xq26	CD40LG 959	CD40LG - CD40 ligand; Mediates B-cell proliferation in the absence of co- stimulus as well as IgE production in the presence of IL-4. Involved in immunoglobulin class switching
8	Fc fragment of IgG, low affinity IIb, receptor (CD32)	1q23	FCG2B [CD32] 2213	Receptor for the Fc region of complexed or aggregated immunoglobulins gamma. Low affinity receptor. Involved in a variety of effector and regulatory functions such as phagocytosis of immune complexes and modulation of antibody production by B- cells. Binding to this receptor results in down-modulation of previous state of cell activation triggered via antigen

	Protein name	Chromo-somal location	Associated Gene Name [Alias] Gene ID	String-db/ Uniprot description
				receptors on B-cells (BCR), T-cells (TCR) or via another Fc receptor. Isoform IIB1 fails to mediate endocytosis or phagocytosis. Isoform IIB2 does not trigger phagocytosis (310 aa)
9	Testican 2	10q22.1	SPOCK2 9806	SPOCK2 - sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2; May participate in diverse steps of neurogenesis. Binds calcium
10	Calcium/calmodulin-dependent protein kinase II beta	7p14.3-p14.1	CAMK2B 816	CaM-kinase II (CAMK2) is a prominent kinase in the central nervous system that may function in long-term potentiation and neurotransmitter release. Member of the NMDAR signalling complex in excitatory synapses, it may regulate NMDAR-dependent potentiation of the AMPAR and synaptic plasticity (By similarity) (666 aa)
11	Checkpoint kinase 2	22q12.1	CHK2 11200	Regulates cell cycle checkpoints and apoptosis in response to DNA damage, particularly to DNA double-strand breaks. Inhibits CDC25C phosphatase by phosphorylation on 'Ser-216', preventing the entry into mitosis. May also play a role in meiosis. Regulates the TP53 tumor suppressor through phosphorylation at 'Thr-18' and 'Ser-20' (586 aa)
12	B-cell Cell lymphoma 2	18q21.3	BCL2 596	Suppresses apoptosis in a variety of cell systems including factor-dependent lymphohematopoietic and neural cells. Regulates cell death by controlling the mitochondrial membrane permeability. Appears to function in a feedback loop system with caspases. Inhibits caspase activity either by preventing the release of cytochrome c from the mitochondria and/or by binding to the apoptosis-activating factor (APAF-1) (239 aa)
13	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4	14q32.13	Kallistatin [SERPINA4] 5267	Inhibits human amidolytic and kininogenase activities of tissue kallikrein. Inhibition is achieved by formation of an equimolar, heat- and SDS-stable complex between the inhibitor and the enzyme, and generation of a small C-terminal fragment of the inhibitor due to cleavage at the reactive site by tissue kallikrein (427 aa)
14	Serine/threonine kinase 16	2q35	STK16 8576	Protein kinase that act on both serine and threonine residues (305 aa)
15	Vasoactive Intestinal Peptide	6q24-q27	VIP 7432	VIP causes vasodilation, lowers arterial blood pressure, stimulates myocardial contractility, increases glycogenolysis and relaxes the smooth muscle of trachea, stomach and gall bladder
16	Carnosine dipeptidase 1	18q22.3	CNDP1 84735	Metallopeptidase M20 family (507 aa)
17	Cathepsin H	15q25.1	CTSH 1512	Important for the overall degradation of proteins in lysosomes (335 aa)

	Protein name	Chromo-somal location	Associated Gene Name [Alias] Gene ID	String-db/ Uniprot description
18	Methyl-CpG binding domain protein 4	3q21.3	MBD4 8930	Mismatch-specific DNA N-glycosylase involved in DNA repair. Has thymine glycosylase activity and is specific for G-T mismatches within methylated and unmethylated CpG sites. Can also remove uracil or 5-fluorouracil in G-U mismatches. Has no lyase activity. Was first identified as methyl-CpG-binding protein (580 aa)
19	Leucine carboxyl methyltransferase 1	16p12.1	LCMT1 51451	Methylates the carboxyl group of the C-terminal leucine residue of protein phosphatase 2A catalytic subunits (PPP2CA) to form alpha-leucine ester residues.
20	Serum amyloid A1	11p15.1	SAA1 6288	Major acute phase reactant. Apolipoprotein of the HDL complex (By similarity) (122 aa)
21	Matrix metallopeptidase 12 (macrophage elastase)	11q22.3	MMP12 4321	May be involved in tissue injury and remodelling. Has significant elastolytic activity. Can accept large and small amino acids at the P1' site, but has a preference for leucine. Aromatic or hydrophobic residues are preferred at the P1 site, with small hydrophobic residues (preferably alanine) occupying P3 (470 aa)
22	Cytohesin 2	19q13.32	PH 9266	Promotes guanine-nucleotide exchange on ARF1, ARF3 and ARF6. Promotes the activation of ARF through replacement of GDP with GTP (399 aa)
23	FAM107B	10p14	83641	family with sequence similarity 107, member B
24	Coagulation factor V (proaccelerin, labile factor)	1q23	F5 2153	Central regulator of hemostasis. It serves as a critical cofactor for the prothrombinase activity of factor Xa that results in the activation of prothrombin to thrombin.
25	Complement component 1, q subcomponent binding protein	17p13.3	C1QBP 708	Not known. Binds to the globular "heads" of C1Q thus inhibiting C1 activation.
26	Esterase D/ formylglutathione hydrolase;	13q14.1-q14.2	ESD 2098	Serine hydrolase involved in the detoxification of formaldehyde (282 aa)
27	Glypican 5	13q32	GPC5 2262	Cell surface proteoglycan that bears heparan sulfate (By similarity) (572 aa)

Table 57: Panel of proteins associated with WMH identified with features selection

(2b) Summary of test statistics from feature selection proteins *versus WMH*

1. CXCL16soluble

```
Residuals:
   Min     1Q Median     3Q    Max
-12825  -5150  -2166   2667  45626

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)     6478.5      728.6   8.892 3.57e-15 ***
data$V2         8615.8     3012.3   2.860  0.00491 **

Residual standard error: 8481 on 134 degrees of freedom
Multiple R-squared:  0.05754,   Adjusted R-squared:  0.0505
F-statistic: 8.181 on 1 and 134 DF,  p-value: 0.004913
============================================================
2. TWEAK

Residuals:
   Min     1Q Median     3Q    Max
-12481  -5572  -2433   2655  46134

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)     6261.3      733.7   8.533 2.69e-14 ***
data$V3       -16463.1     6696.5  -2.458   0.0152 *

Residual standard error: 8546 on 134 degrees of freedom
Multiple R-squared:  0.04316,   Adjusted R-squared:  0.03602
F-statistic: 6.044 on 1 and 134 DF,  p-value: 0.01523
============================================================
3. HPV E7 Type 16

Residuals:
   Min     1Q Median     3Q    Max
-13685  -5603  -2403   2631  42735

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)     6342.6      729.2   8.699 1.06e-14 ***
data$V4        10210.8     3742.4   2.728  0.00722 **

Residual standard error: 8503 on 134 degrees of freedom
Multiple R-squared:  0.05263,   Adjusted R-squared:  0.04556
F-statistic: 7.444 on 1 and 134 DF,  p-value: 0.007218
============================================================
4. Macrophage mannose receptor

Residuals:
   Min     1Q Median     3Q    Max
 -9471  -5427  -2697   3209  45612

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)     6479.3      729.6   8.880 3.82e-15 ***
data$V5         7505.3     2687.8   2.792    0.006 **

Residual standard error: 8493 on 134 degrees of freedom
Multiple R-squared:  0.05499,   Adjusted R-squared:  0.04794
F-statistic: 7.797 on 1 and 134 DF,  p-value: 0.005998
============================================================
5. GDF 11

Residuals:
   Min     1Q Median     3Q    Max
-10404  -5074  -2219   2520  43700

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6196.4      717.4   8.637  1.5e-14 ***
```

```
data$V6      -9878.5     2775.7  -3.559 0.000516 ***


Residual standard error: 8350 on 134 degrees of freedom
Multiple R-squared:  0.08636,   Adjusted R-squared:  0.07954
F-statistic: 12.67 on 1 and 134 DF,  p-value: 0.0005157
============================================================
6. Alkaline phosphatase bone

Residuals:
   Min     1Q Median     3Q    Max
 -8172  -5796  -2771   2160  47934

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6391.4      738.8   8.651 1.39e-14 ***
data$V7      -2211.7     1125.4  -1.965   0.0514 .


Residual standard error: 8613 on 134 degrees of freedom
Multiple R-squared:  0.02802,   Adjusted R-squared:  0.02076
F-statistic: 3.863 on 1 and 134 DF,  p-value: 0.05144
============================================================
7. IgM

Residuals:
   Min     1Q Median     3Q    Max
 -8517  -5610  -2347   2973  47230

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6160.5      743.8   8.283 1.09e-13 ***
data$V8      -2400.4     1176.9  -2.040   0.0434 *


Residual standard error: 8604 on 134 degrees of freedom
Multiple R-squared:  0.03011,   Adjusted R-squared:  0.02287
F-statistic:  4.16 on 1 and 134 DF,  p-value: 0.04335
------------------------------------------------------------
============================================================
8. FCG2B

Residuals:
   Min     1Q Median     3Q    Max
-11034  -5374  -2419   2224  44452

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    6466.8      730.0   8.859  4.3e-15 ***
data$V9        2422.8      879.7   2.754   0.0067 **

Residual standard error: 8499 on 134 degrees of freedom
Multiple R-squared:  0.05357,   Adjusted R-squared:  0.04651
F-statistic: 7.585 on 1 and 134 DF,  p-value: 0.006703
============================================================
9. Testican 2

Residuals:
   Min     1Q Median     3Q    Max
 -9849  -5315  -2374   3911  44280

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6459.6      728.2   8.871 4.03e-15 ***
data$V10    -11043.2     3859.1  -2.862  0.00489 **
```

```
Residual standard error: 8481 on 134 degrees of freedom
Multiple R-squared:  0.05759,   Adjusted R-squared:  0.05056
F-statistic: 8.189 on 1 and 134 DF,  p-value: 0.004893
================================================================
10. CAMK2B

Residuals:
   Min     1Q Median     3Q    Max
 -8612  -5807  -2526   2379  47167

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)      6285.8      734.7   8.555 2.38e-14 ***
data$V11    -12408.4     5284.4  -2.348   0.0203 *

Residual standard error: 8562 on 134 degrees of freedom
Multiple R-squared:  0.03952,   Adjusted R-squared:  0.03235
F-statistic: 5.514 on 1 and 134 DF,  p-value: 0.02033

================================================================
11. Chk2

Residuals:
   Min     1Q Median     3Q    Max
 -9407  -5509  -2561   2047  48820

Coefficients:
          Estimate Std. Error t value Pr(>|t|)
(Intercept)   6405.1      716.5   8.940 2.73e-15 ***
data$V12      5629.9     1589.4   3.542 0.000547 ***


Residual standard error: 8354 on 134 degrees of freedom
Multiple R-squared:  0.08562,   Adjusted R-squared:  0.07879
F-statistic: 12.55 on 1 and 134 DF,  p-value: 0.0005466
================================================================
12. Bcl2

Residuals:
   Min     1Q Median     3Q    Max
 -8647  -5546  -3077   2557  47158

Coefficients:
          Estimate Std. Error t value Pr(>|t|)
(Intercept)   6240.1      740.5   8.427 4.88e-14 ***
data$V13      8982.5     4511.4   1.991   0.0485 *


Residual standard error: 8610 on 134 degrees of freedom
Multiple R-squared:  0.02873,   Adjusted R-squared:  0.02149
F-statistic: 3.964 on 1 and 134 DF,  p-value: 0.0485
================================================================
13. Kallistatin

Residuals:
   Min     1Q Median     3Q    Max
-11278  -5367  -2481   2585  41953

Coefficients:
          Estimate Std. Error t value Pr(>|t|)
(Intercept)   6318.8      731.1   8.643 1.46e-14 ***
data$V14     -8748.9     3370.3  -2.596   0.0105 *


Residual standard error: 8525 on 134 degrees of freedom
Multiple R-squared:  0.04788,   Adjusted R-squared:  0.04077
F-statistic: 6.738 on 1 and 134 DF,  p-value: 0.01049
```

```
============================================================
14. STK16

Residuals:
   Min     1Q Median     3Q    Max
-10717  -5319  -2849   2485  45890

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)     6370.5      725.7   8.778 6.79e-15 ***
data$V15       -9476.4     3194.8  -2.966  0.00357 **

Residual standard error: 8463 on 134 degrees of freedom
Multiple R-squared:  0.06161,   Adjusted R-squared:  0.05461
F-statistic: 8.798 on 1 and 134 DF,  p-value: 0.003571

============================================================
15. Vasoactive Intestinal Peptide

Residuals:
   Min     1Q Median     3Q    Max
 -7892  -6004  -2947   3478  46274

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6411.4      745.3   8.602 1.83e-14 ***
data$V16     -7786.5     5761.5  -1.351    0.179


Residual standard error: 8677 on 134 degrees of freedom
Multiple R-squared:  0.01345,   Adjusted R-squared:  0.006085
F-statistic: 1.827 on 1 and 134 DF,  p-value: 0.1788
============================================================
16. CNDP1

Residuals:
   Min     1Q Median     3Q    Max
 -9709  -5129  -2745   2800  43986

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6381.1      729.1   8.751 7.91e-15 ***
data$V17     -4120.4     1507.5  -2.733  0.00712 **


Residual standard error: 8502 on 134 degrees of freedom
Multiple R-squared:  0.05281,   Adjusted R-squared:  0.04574
F-statistic: 7.471 on 1 and 134 DF,  p-value: 0.007116
============================================================
17. Cathepsin H

Residuals:
   Min     1Q Median     3Q    Max
 -9703  -4840  -2701   2647  45699

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6401.5      707.3   9.050 1.46e-15 ***
data$V18     11962.0     2958.7   4.043 8.86e-05 ***


Residual standard error: 8248 on 134 degrees of freedom
Multiple R-squared:  0.1087,    Adjusted R-squared:  0.1021
F-statistic: 16.35 on 1 and 134 DF,  p-value: 8.856e-05
============================================================
18. MBD4
```

```
Residuals:
   Min     1Q Median     3Q    Max
 -7237  -5926  -2869   3396  46553

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6344.3      741.9   8.552 2.43e-14 ***
data$V19     -6175.2     3803.7  -1.623    0.107


Residual standard error: 8652 on 134 degrees of freedom
Multiple R-squared:  0.01929,   Adjusted R-squared:  0.01197
F-statistic: 2.636 on 1 and 134 DF,  p-value: 0.1068
============================================================
19. LCMT1

Residuals:
   Min     1Q Median     3Q    Max
 -7796  -5843  -2760   2820  47361

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6365.2      741.1   8.589 1.97e-14 ***
data$V20     -5582.2     3261.0  -1.712   0.0893 .


Residual standard error: 8642 on 134 degrees of freedom
Multiple R-squared:  0.0214,    Adjusted R-squared:  0.0141
F-statistic:  2.93 on 1 and 134 DF,  p-value: 0.08925
============================================================
20. SAA

Residuals:
   Min     1Q Median     3Q    Max
-12727  -5190  -2460   3519  40993

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6497.6      730.1   8.899 3.43e-15 ***
data$V21      1333.5      478.4   2.788  0.00608 **


Residual standard error: 8493 on 134 degrees of freedom
Multiple R-squared:  0.05481,   Adjusted R-squared:  0.04776
F-statistic: 7.771 on 1 and 134 DF,  p-value: 0.006081
============================================================
21. MMP 12

Residuals:
   Min     1Q Median     3Q    Max
-10216  -5129  -2269   2356  46413

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    6352.6      719.7   8.827 5.17e-15 ***
data$V22      11725.0     3506.5   3.344  0.00107 **

Residual standard error: 8393 on 134 degrees of freedom
Multiple R-squared:  0.07702,   Adjusted R-squared:  0.07013
F-statistic: 11.18 on 1 and 134 DF,  p-value: 0.001072

============================================================
22. PH

Residuals:
   Min     1Q Median     3Q    Max
```

```
-12876  -4864  -2321   3429  35890

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6197.2      713.1   8.691 1.11e-14 ***
data$V23      3512.5      926.1   3.793 0.000224 ***


Residual standard error: 8302 on 134 degrees of freedom
Multiple R-squared:  0.09695,   Adjusted R-squared:  0.09021
F-statistic: 14.39 on 1 and 134 DF,  p-value: 0.0002244
================================================================
23. FAM107B

Residuals:
   Min     1Q Median     3Q    Max
-11042  -4911  -2605   2559  38846

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6100.2      721.4   8.456 4.15e-14 ***
data$V24      6766.7     1953.5   3.464 0.000715 ***


Residual standard error: 8370 on 134 degrees of freedom
Multiple R-squared:  0.08218,   Adjusted R-squared:  0.07533
F-statistic:    12 on 1 and 134 DF,  p-value: 0.0007155
================================================================
24. Coagulation Factor V

Residuals:
   Min     1Q Median     3Q    Max
 -9990  -5226  -2124   2795  45823

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6129.2      737.7   8.309 9.44e-14 ***
data$V25      7476.6     2990.1   2.500   0.0136 *


Residual standard error: 8539 on 134 degrees of freedom
Multiple R-squared:  0.04458,   Adjusted R-squared:  0.03745
F-statistic: 6.252 on 1 and 134 DF,  p-value: 0.01361
================================================================
25. C1QBP

Residuals:
   Min     1Q Median     3Q    Max
-11983  -5453  -2046   2127  48230

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6496.7      726.5   8.943 2.68e-15 ***
data$V26    -20017.4     6635.8  -3.017  0.00306 **


Residual standard error: 8454 on 134 degrees of freedom
Multiple R-squared:  0.06359,   Adjusted R-squared:  0.0566
F-statistic:   9.1 on 1 and 134 DF,  p-value: 0.00306
================================================================
26. Esterase D

Residuals:
   Min     1Q Median     3Q    Max
 -7996  -5889  -2371   2444  47168

Coefficients:
```

```
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6382.4      739.2   8.634 1.53e-14 ***
data$V27     -5393.6     2811.7  -1.918   0.0572 .


Residual standard error: 8619 on 134 degrees of freedom
Multiple R-squared:  0.02673,   Adjusted R-squared:  0.01946
F-statistic:  3.68 on 1 and 134 DF,  p-value: 0.0572
============================================================
27. GPC5

Residuals:
   Min     1Q Median     3Q    Max
-13395  -5196  -2921   2339  40049

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   6297.3      693.6   9.079 1.23e-15 ***
data$V28      9235.1     1952.8   4.729 5.64e-06 ***


Residual standard error: 8087 on 134 degrees of freedom
Multiple R-squared:  0.143,       Adjusted R-squared:  0.1366
F-statistic: 22.37 on 1 and 134 DF,  p-value: 5.636e-06
============================================================

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Table 58: Test-statistic from selected features: proteins versus WMH

(2c) Regression - WMH volume versus protein concentration

In AD, MCI & controls

```
=== Run information ===

Scheme:weka.classifiers.meta.CVParameterSelection -P "C 0.0 1.0 1.0" -P "R -5.99999999 5.0E-4 5.00000001" -X 10 -S 2 -W
weka.classifiers.functions.LinearRegression -- -S 0 -R 1.0E-8
Relation:     wml.rna_status-weka.filters.unsupervised.attribute.Reorder-R2,..,1017,1-
weka.filters.supervised.attribute.AttributeSelection-Eweka.attributeSelection.CfsSubsetEval-Sweka.attributeSelection.BestFirst -D
1 -N 5
Instances:    136
Attributes:   28
              CXCL16__soluble
              TWEAK
              HPV_E7_Type_16
              Macrophage_mannose_receptor
              GDF_11
              Alkaline_phosphatase__bone
              IgM
              FCG2B
              Testican_2
              CAMK2B
              Chk2
              Bcl_2
              Kallistatin
              STK16
              Vasoactive_Intestinal_Peptide
              CNDP1
              Cathepsin_H
```

```
            MBD4
            LCMT1
            SAA
            MMP_12
            PH
            FAM107B
            Coagulation_Factor_V
            C1QBP
            Esterase_D
            GPC5
            WMH
Test mode:evaluate on training data

=== Classifier model (full training set) ===

Cross-validated Parameter selection.
Classifier: weka.classifiers.functions.LinearRegression
Cross-validation Parameter: '-C' ranged from 0.0 to 1.0 with 1.0 steps
Cross-validation Parameter: '-R' ranged from -5.99999999 to 5.0E-4 with 5.00000001 steps
Classifier Options: -C 0 -R 4.999849987497029E-4 -S 0


Linear Regression Model

WMH =

   3820.8925 * CXCL16__soluble +
  -6463.3624 * GDF_11 +
  -1644.9321 * Alkaline_phosphatase__bone +
  -1217.2272 * IgM +
  -4849.6614 * Testican_2 +
   6061.9888 * Chk2 +
   5828.7514 * Bcl_2 +
  -4397.259  * Kallistatin +
  -6794.0243 * Vasoactive_Intestinal_Peptide +
  -1917.0122 * CNDP1 +
   3635.0683 * Cathepsin_H +
  -4192.7536 * MBD4 +
  -6090.1537 * LCMT1 +
    761.025  * SAA +
   1564.6049 * PH +
   3433.2353 * FAM107B +
   6016.1942 * Coagulation_Factor_V +
  -9031.0426 * C1QBP +
  -5234.766  * Esterase_D +
   3479.6264 * GPC5 +
   6110.9159

Time taken to build model: 0.12 seconds

=== Evaluation on training set ===
=== Summary ===

Correlation coefficient                  0.8029
Mean absolute error                   3946.3203
Root mean squared error               5169.2549
Relative absolute error                 65.7989 %
Root relative squared error             59.6101 %
Total Number of Instances                136



Time taken to build model: 0 seconds

=== Cross-validation  training set ===

Correlation coefficient                  0.4236
```

Mean absolute error 5978.64
Root mean squared error 8390.7211
Relative absolute error 99.1771 %
Root relative squared error 96.2715 %
Total Number of Instances 136

=== Re-evaluation on test set ===

User supplied test set
Relation: wml.rna_status-weka.filters.unsupervised.attribute.Remove-R2....Reorder-R2,..
Instances: unknown (yet). Reading incrementally
Attributes: 28

=== Summary ===

Correlation coefficient 0.2953
Mean absolute error 7265.2504
Root mean squared error 10126.5349
Total Number of Instances 46

Table 59: WMH versus protein concentration protocol in AD, MCI, and controls

In AD

=== Run information ===

Scheme:weka.classifiers.meta.CVParameterSelection -P "C 0.0 1.0 1.0" -P "R -5.99999999 5.0E-4 5.00000001" -X 10 -S 1 -W weka.classifiers.functions.LinearRegression -- -S 0 -R 1.0E-8
Relation: wml.rna_status-weka.filters.unsupervised.attribute.Remove-R2-..-1017
Instances: 59
Attributes: 28
WMH
CXCL16__soluble
TWEAK
HPV_E7_Type_16
Macrophage_mannose_receptor
GDF_11
Alkaline_phosphatase__bone
IgM
FCG2B
Testican_2
CAMK2B
Chk2
Bcl_2
Kallistatin
STK16
Vasoactive_Intestinal_Peptide
CNDP1
Cathepsin_H
MBD4
LCMT1
SAA
MMP_12
PH
FAM107B
Coagulation_Factor_V
C1QBP
Esterase_D
GPC5
Test mode:evaluate on training data

=== Classifier model (full training set) ===

```
Cross-validated Parameter selection.
Classifier: weka.classifiers.functions.LinearRegression
Cross-validation Parameter: '-C' ranged from 0.0 to 1.0 with 1.0 steps
Cross-validation Parameter: '-R' ranged from -5.99999999 to 5.0E-4 with 5.00000001 steps
Classifier Options: -C 0 -R -2.999750002500625 -S 0


Linear Regression Model

WMH =

 -11324.4264 * GDF_11 +
  -7311.199  * Testican_2 +
   7906.4421 * Chk2 +
  25193.4117 * Bcl_2 +
  -7727.4841 * Kallistatin +
  17206.7093 * STK16 +
   4576.3687 * CNDP1 +
  13432.8211 * Cathepsin_H +
  -1130.5637 * SAA +
   9192.2897 * MMP_12 +
   3772.0682 * FAM107B +
 -14788.0627 * C1QBP +
 -19285.4322 * GPC5 +
   5350.1558

Time taken to build model: 0.04 seconds

=== Evaluation on training set ===
=== Summary ===

Correlation coefficient                  0.8695
Mean absolute error                   3825.124
Root mean squared error               4565.1257
Relative absolute error                 54.2825 %
Root relative squared error             49.8264 %
Total Number of Instances              59

=== Cross-validation ===
=== Summary ===

Correlation coefficient                  0.3399
Mean absolute error                   7514.7552
Root mean squared error              12966.1292
Relative absolute error                104.0709 %
Root relative squared error            138.6872 %
Total Number of Instances              59


=== Re-evaluation on test set ===

User supplied test set
Relation:     wml.rna_status-weka.filters.unsupervised.attribute.Remove-R2-..-1017
Instances:     unknown (yet). Reading incrementally
Attributes:   28

=== Summary ===

Correlation coefficient                  0.1711
Mean absolute error                   7330.0636
Root mean squared error               9574.7467
Total Number of Instances              30
```

Table 60: WMH versus protein concentration protocol in AD

(3a) Panel of mRNA transcripts associated with the rate of cognitive decline identified with features selection

	Full name	Chromo-somal location	Gene Name [Alias] Gene ID	Description (String-db, genecards.org)
1	Amyotrophic Lateral Sclerosis 2 Chromosomal Region Candidate Gene 2	2q33.1	ALS2CR2 [STRADB] 55437	STRADB - STE20-related kinase adaptor beta; Pseudokinase which, in complex with CAB39, binds to and activates STK11. Relocates STK11 from the nucleus to the cytoplasm. Plays an essential role in STK11-mediated G1 cell cycle arrest
2	Bromodomain containing 2	6p21.3	BRD2 6046	associates with transcription complexes and with acetylated chromatin during mitosis, and it selectively binds to the acetylated lysine-12 residue of histone H4 via its two bromodomains. The gene maps to the major histocompatability complex (MHC) class II region on chromosome 6p21.3, but sequence comparison suggests that the protein is not involved in the immune response. This gene has been implicated in juvenile myoclonic epilepsy, a common form of epilepsy that becomes apparent in adolescence.
3	Chemokine (C-C motif) receptor 6	6q27	CCR6A 1235	Chemokine (C-C motif) receptor 6; Receptor for a C-C type chemokine. Binds to MIP-3- alpha/LARC and subsequently transduces a signal by increasing the intracellular calcium ions level
4	CD81 molecule	11p15.5	CD81 [TSPAN28] 975	May play an important role in the regulation of lymphoma cell growth. Interacts with a 16-kDa Leu-13 protein to form a complex possibly involved in signal transduction. May acts a the viral receptor for HCV
5	Charged multivesicular body protein 6	17q25.3	CHMP6 79643	chromatin modifying protein 6; Probable core component of the endosomal sorting required for transport complex III (ESCRT-III) which is involved in multivesicular bodies (MVBs) formation and sorting of endosomal cargo proteins into MVBs. MVBs contain intraluminal vesicles (ILVs) that are generated by invagination and scission from the limiting membrane of the endosome and mostly are delivered to lysosomes enabling degradation of membrane proteins, such as stimulated growth factor receptors, lysosomal enzymes and lipids.
6	CCR4-NOT transcription complex, subunit 2	12q15	CNOT2 4848	The CCR4-NOT complex functions as general transcription regulation complex
7	Cortactin	11q13	CTTN	May contribute to the organization of cell structure. The

	Full name	Chromo-somal location	Gene Name [Alias] Gene ID	Description (String-db, genecards.org)
			2017	SH3 motif may function as a binding region to cytoskeleton. Tyrosine phosphorylation in transformed cells may contribute to cellular growth regulation and transformation
8	Dynactin 5	16p12.2	DCTN5 84516	This gene encodes a subunit of dynactin, a component of the cytoplasmic dynein motor machinery involved in minus-end-directed transport. The encoded protein is a component of the pointed-end subcomplex and is thought to bind membranous cargo.
9	Dual specificity phosphatase 3	17q21	DUSP3 1845	activity both toward tyrosine-protein phosphate as well as with serine-protein phosphate
10	Granulysin	2p11.2	GNLY 10578	Antimicrobial protein that kills intracellular pathogens. Active against a broad range of microbes, including Gram-positive and Gram-negative bacteria, fungi, and parasites. Kills Mycobacterium tuberculosis
11	Glycosylphosphatidylinositol anchor attachment 1	8q24.3	GPAA1 8733	Essential for GPI-anchoring of precursor proteins but not for GPI synthesis. Acts before or during formation of the carbonyl intermediate
12	Hemoglobin, alpha 2	16p13.3	HBA2A 3040	Involved in oxygen transport from the lung to the various peripheral tissues
13	Major histocompatibility complex, class I, B	6p21.3	HLAB 3106	B-48 alpha chain Precursor (MHC class I antigen B*48) (Bw-48); Involved in the presentation of foreign antigens to the immune system
14	Chromosome 1 open reading frame 132	1q32.2	HS.445414 100128537	Chromosome 1 open reading frame 132
15	Uncharacterized protein	19q13.11	KIAA0355 9710	Diseases associated with KIAA0355 include bipolar disorder.
16	Poly(A) binding protein, cytoplasmic 1	Chr16/exon15	PABPC1A 606498	RNA recognition motif-containing protein RRM; Binds the poly(A) tail of mRNA. Appears to be an important mediator of the multiple roles of the poly(A) tail in mRNA biogenesis, stability and translation
17	Periphilin 1	12q12	PPHLN1 51535	Involved in epithelial differentiation and contributes to epidermal integrity and barrier formation
18	PRP40 pre-mRNA processing factor 40 homolog A	2q23.3	PRPF40A 55660	Binds to WASL/N-WASP and suppresses its translocation from the nucleus to the cytoplasm, thereby inhibiting its cytoplasmic function. May be involved in pre-mRNA splicing
19	Cytohesin 1	2q11.2	PSCD1A [CYTIP]	The protein encoded by this gene contains 2 leucine zipper domains and a putative C-terminal nuclear targeting signal, but does not have any hydrophobic regions. This protein is expressed weakly in resting NK and T cells.
20	RALBP1 associated Eps	Xp22.2	REPS2 9185	Involved in growth factor signaling through its influence on the Ral signaling pathway

	Full name	Chromo-somal location	Gene Name [Alias] Gene ID	Description (String-db, genecards.org)
	domain containing 2			
21	RNA, 7SL, cytoplasmic 1	14q21.3	RN7SL1 6029	The signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein complex that mediates cotranslational insertion of secretory proteins into the lumen of the endoplasmic reticulum.
22	Ring finger protein 7	3q22-q24	RNF7 9616	Ring finger protein 7; Probable component of the SCF (SKP1-CUL1-F-box protein) E3 ubiquitin ligase complex which mediates the ubiquitination and subsequent proteasomal degradation of target proteins involved in cell cycle progression, signal transduction and transcription. Through the RING-type zinc finger, seems to recruit the E2 ubiquitination enzyme to the complex and brings it into close proximity to the substrate. Promotes the neddylation of CUL5 via its interaction with UBE2F. May play a role in protecting cells from apoptosis induced by redox agents
23	Solute carrier family 5 (sodium/ glucose cotransporter), member 6	2p23	SLC5A6 8884	Transports pantothenate, biotin and lipoate in the presence of sodium
24	Transmembrane protein 137	11q13.1	TMEM137 [COAA, RBM 14] 84972	Isoform 1 may function as a nuclear receptor coactivator, enhancing transcription through other coactivators such as NCOA6 and CITED1. Isoform 2, functions as a transcriptional repressor, modulating transcriptional activities of coactivators including isoform 1, NCOA6 and CITED1
25	Transmembrane protein 59	1p32.3	TMEM59 9528	Diseases associated with TMEM59 include liver disease, and Alzheimer's disease. Annotations related to this gene include endopeptidase activity.
26	Vaccinia related kinase 3		VRK3A	required for catalysis
27	Yip1 interacting factor homolog A	11q13	YIF1A 10897	Possible role in transport between endoplasmic reticulum and Golgi

Table 61: Description of cognitive decline-associated mRNAs from feature selection

(3b) Summary of test statistics *from feature mRNA versus rate of cognitive decline*

1. ALS2CR2

Residuals:

Min	1Q	Median	3Q	Max
-0.0082182	-0.0022373	-0.0005406	0.0019186	0.0121887

```
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0038628  0.0005907    6.54 5.42e-08 ***
data$V1     0.0130603  0.0035108    3.72 0.000561 ***


Residual standard error: 0.003908 on 44 degrees of freedom
Multiple R-squared:  0.2393,    Adjusted R-squared:  0.222
F-statistic: 13.84 on 1 and 44 DF,  p-value: 0.0005614
============================================================
2. BRD2

Residuals:
      Min        1Q    Median        3Q       Max
-0.0095582 -0.0027261 -0.0004228  0.0018599  0.0129412

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0029644  0.0006513   4.552 4.17e-05 ***
data$V2     -0.0131997  0.0058109  -2.272   0.0281 *


Residual standard error: 0.004239 on 44 degrees of freedom
Multiple R-squared:  0.105,      Adjusted R-squared:  0.08462
F-statistic:  5.16 on 1 and 44 DF,  p-value: 0.02806
============================================================
3. CCR6A


Residuals:
      Min        1Q    Median        3Q       Max
-0.0067004 -0.0028614 -0.0003312  0.0018301  0.0140134

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0031473  0.0006098   5.162 5.65e-06 ***
data$V3     0.0156003  0.0053347   2.924  0.00544 **


Residual standard error: 0.0041 on 44 degrees of freedom
Multiple R-squared:  0.1627,    Adjusted R-squared:  0.1437
F-statistic: 8.552 on 1 and 44 DF,  p-value: 0.005438
============================================================
4. CD81

Residuals:
      Min        1Q    Median        3Q       Max
-0.0110388 -0.0025620 -0.0002307  0.0017755  0.0119764

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0024982  0.0006429   3.886 0.000339 ***
data$V4     -0.0057519  0.0016938  -3.396 0.001460 **


Residual standard error: 0.003989 on 44 degrees of freedom
Multiple R-squared:  0.2077,    Adjusted R-squared:  0.1897
F-statistic: 11.53 on 1 and 44 DF,  p-value: 0.00146
============================================================
5. CHMP6

Residuals:
      Min        1Q    Median        3Q       Max
-0.0085210 -0.0030074 -0.0009156  0.0028689  0.0138606

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
```

```
(Intercept)  0.0033083  0.0006175   5.357 2.94e-06 ***
data$V5     -0.0111040  0.0043663  -2.543   0.0146 *


Residual standard error: 0.004184 on 44 degrees of freedom
Multiple R-squared:  0.1281,    Adjusted R-squared:  0.1083
F-statistic: 6.467 on 1 and 44 DF,  p-value: 0.01458
============================================================
6. CNOT2

Residuals:
      Min        1Q    Median        3Q       Max
-0.0074426 -0.0029448  0.0000664  0.0019593  0.0122247

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0036227  0.0006163   5.878  5.1e-07 ***
data$V6     -0.0197117  0.0071450  -2.759  0.00842 **


Residual standard error: 0.004137 on 44 degrees of freedom
Multiple R-squared:  0.1475,    Adjusted R-squared:  0.1281
F-statistic: 7.611 on 1 and 44 DF,  p-value: 0.008421
============================================================
7. CTTN

Residuals:
      Min        1Q    Median        3Q       Max
-0.0097493 -0.0021147 -0.0001392  0.0017233  0.0095500

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0036828  0.0006124   6.014 3.22e-07 ***
data$V7     0.0072192  0.0024491   2.948  0.00511 **


Residual standard error: 0.004095 on 44 degrees of freedom
Multiple R-squared:  0.1649,    Adjusted R-squared:  0.1459
F-statistic: 8.689 on 1 and 44 DF,  p-value: 0.005107
============================================================
8. DCTN5

Residuals:
      Min        1Q    Median        3Q       Max
-0.0081032 -0.0024563 -0.0004236  0.0017436  0.0162222

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0032876  0.0006313   5.207 4.85e-06 ***
data$V8     -0.0101979  0.0048498  -2.103   0.0412 *


Residual standard error: 0.004271 on 44 degrees of freedom
Multiple R-squared:  0.09131,   Adjusted R-squared:  0.07066
F-statistic: 4.422 on 1 and 44 DF,  p-value: 0.04124
============================================================
9. DUSP3

Residuals:
      Min        1Q    Median        3Q       Max
-0.0087018 -0.0025532 -0.0005423  0.0010816  0.0136325

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0033680  0.0006353   5.302 3.54e-06 ***
data$V9     -0.0099663  0.0052562  -1.896   0.0645 .
```

```
Residual standard error: 0.004308 on 44 degrees of freedom
Multiple R-squared:  0.07554,   Adjusted R-squared:  0.05453
F-statistic: 3.595 on 1 and 44 DF,  p-value: 0.06453
================================================================
10. GNLY

Residuals:
      Min        1Q    Median        3Q       Max
-0.0082926 -0.0025632 -0.0004306  0.0015250  0.0134168

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0039799  0.0006163   6.457 7.17e-08 ***
data$V10    0.0072559  0.0021663   3.349  0.00167 **


Residual standard error: 0.004 on 44 degrees of freedom
Multiple R-squared:  0.2032,    Adjusted R-squared:  0.1851
F-statistic: 11.22 on 1 and 44 DF,  p-value: 0.001669
================================================================
11. GPAA1

Residuals:
      Min        1Q    Median        3Q       Max
-0.0072480 -0.0030077 -0.0004425  0.0017410  0.0118674

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0031190  0.0006073   5.136 6.15e-06 ***
data$V11    -0.0121484  0.0040165  -3.025  0.00415 **


Residual standard error: 0.004077 on 44 degrees of freedom
Multiple R-squared:  0.1721,    Adjusted R-squared:  0.1533
F-statistic: 9.148 on 1 and 44 DF,  p-value: 0.004145
================================================================
12. HBA2A

Residuals:
      Min        1Q    Median        3Q       Max
-0.0077682 -0.0023379 -0.0007443  0.0019249  0.0143548

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.003342   0.000585   5.713 8.91e-07 ***
data$V12    -0.017587   0.005049  -3.484  0.00113 **


Residual standard error: 0.003967 on 44 degrees of freedom
Multiple R-squared:  0.2162,    Adjusted R-squared:  0.1984
F-statistic: 12.14 on 1 and 44 DF,  p-value: 0.001132
================================================================
13. HLAB

Residuals:
      Min        1Q    Median        3Q       Max
-0.0073274 -0.0030150 -0.0003404  0.0024477  0.0140755

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0033052  0.0006256   5.283 3.77e-06 ***
data$V13    0.0121420  0.0053237   2.281   0.0275 *


Residual standard error: 0.004237 on 44 degrees of freedom
Multiple R-squared:  0.1057,    Adjusted R-squared:  0.0854
```

```
F-statistic: 5.202 on 1 and 44 DF,  p-value: 0.02746
============================================================
14. HS.445414

Residuals:
      Min        1Q    Median        3Q       Max
-0.0088409 -0.0016793 -0.0006719  0.0019849  0.0136966

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0031836  0.0006199   5.136 6.15e-06 ***
data$V14    0.0094169  0.0036279   2.596   0.0128 *


Residual standard error: 0.004173 on 44 degrees of freedom
Multiple R-squared:  0.1328,    Adjusted R-squared:  0.1131
F-statistic: 6.737 on 1 and 44 DF,  p-value: 0.01278
============================================================
15. KIAA0355

Residuals:
      Min        1Q    Median        3Q       Max
-0.0096253 -0.0025727 -0.0007016  0.0020459  0.0109879

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0034726  0.0006055   5.735 8.27e-07 ***
data$V15    -0.0095739  0.0032793  -2.920  0.00551 **


Residual standard error: 0.004101 on 44 degrees of freedom
Multiple R-squared:  0.1623,    Adjusted R-squared:  0.1432
F-statistic: 8.524 on 1 and 44 DF,  p-value: 0.005508
============================================================
16. PABPC1A

Residuals:
      Min        1Q    Median        3Q       Max
-0.0087138 -0.0028665 -0.0006023  0.0017318  0.0118942

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0032158  0.0006135   5.242 4.32e-06 ***
data$V16    0.0055342  0.0020179   2.743  0.00878 **


Residual standard error: 0.004141 on 44 degrees of freedom
Multiple R-squared:  0.146,     Adjusted R-squared:  0.1266
F-statistic: 7.522 on 1 and 44 DF,  p-value: 0.008783
============================================================
17. PPHLN1

Residuals:
      Min        1Q    Median        3Q       Max
-0.0098591 -0.0027344 -0.0004713  0.0023597  0.0130339

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0032582  0.0006141   5.306 3.49e-06 ***
data$V17    0.0101081  0.0037629   2.686   0.0102 *


Residual standard error: 0.004153 on 44 degrees of freedom
Multiple R-squared:  0.1409,    Adjusted R-squared:  0.1214
F-statistic: 7.216 on 1 and 44 DF,  p-value: 0.01016
============================================================
18. PRPF40A
```

```
Residuals:
     Min       1Q   Median       3Q      Max
-0.009820 -0.002485 -0.001213  0.002292  0.014889

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0030994  0.0006298   4.921 1.25e-05 ***
data$V18    0.0130702  0.0053070   2.463   0.0178 *


Residual standard error: 0.004201 on 44 degrees of freedom
Multiple R-squared:  0.1212,    Adjusted R-squared:  0.1012
F-statistic: 6.066 on 1 and 44 DF,  p-value: 0.01777
============================================================
19. PSCD1A

Residuals:
      Min        1Q    Median        3Q       Max
-0.0098846 -0.0027833 -0.0002113  0.0012543  0.0137247

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0037248  0.0006781   5.493 1.87e-06 ***
data$V19    -0.0076177  0.0047949  -1.589    0.119


Residual standard error: 0.004358 on 44 degrees of freedom
Multiple R-squared:  0.05425,   Adjusted R-squared:  0.03276
F-statistic: 2.524 on 1 and 44 DF,  p-value: 0.1193
============================================================
20. REPS2

Residuals:
      Min        1Q    Median        3Q       Max
-0.0086229 -0.0024903 -0.0001709  0.0015716  0.0113907

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0039773  0.0006465   6.153 2.01e-07 ***
data$V20    -0.0113536  0.0040937  -2.773  0.00811 **


Residual standard error: 0.004134 on 44 degrees of freedom
Multiple R-squared:  0.1488,    Adjusted R-squared:  0.1295
F-statistic: 7.692 on 1 and 44 DF,  p-value: 0.008106
============================================================
21. RN7SL1

Residuals:
      Min        1Q    Median        3Q       Max
-0.0091788 -0.0022480 -0.0007017  0.0019973  0.0120271

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0041607  0.0006261   6.645  3.8e-08 ***
data$V21    0.0114198  0.0032744   3.488  0.00112 **


Residual standard error: 0.003966 on 44 degrees of freedom
Multiple R-squared:  0.2166,    Adjusted R-squared:  0.1988
F-statistic: 12.16 on 1 and 44 DF,  p-value: 0.001118
============================================================
22. RNF7

Residuals:
      Min        1Q    Median        3Q       Max
```

```
-0.0082629 -0.0024963 -0.0002965  0.0013276  0.0142565

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0030286  0.0006266   4.833 1.67e-05 ***
data$V22    -0.0165915  0.0061985  -2.677   0.0104 *


Residual standard error: 0.004155 on 44 degrees of freedom
Multiple R-squared:   0.14,       Adjusted R-squared:  0.1205
F-statistic: 7.165 on 1 and 44 DF,  p-value: 0.01041
================================================================
23. SLC5A6


Residuals:
      Min        1Q    Median        3Q       Max
-0.0085848 -0.0018501 -0.0001485  0.0018678  0.0117469

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0038243  0.0005839   6.549 5.25e-08 ***
data$V23    0.0092503  0.0024172   3.827 0.000406 ***


Residual standard error: 0.003881 on 44 degrees of freedom
Multiple R-squared:  0.2497,    Adjusted R-squared:  0.2327
F-statistic: 14.64 on 1 and 44 DF,  p-value: 0.0004065
================================================================
24. TMEM137

Residuals:
      Min        1Q    Median        3Q       Max
-0.0091566 -0.0020866 -0.0004213  0.0014961  0.0140435

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0033892  0.0006319   5.364 2.88e-06 ***
data$V24    -0.0114025  0.0056282  -2.026   0.0489 *


Residual standard error: 0.004286 on 44 degrees of freedom
Multiple R-squared:  0.08532,   Adjusted R-squared:  0.06454
F-statistic: 4.104 on 1 and 44 DF,  p-value: 0.04886
================================================================
25. TMEM59

Residuals:
      Min        1Q    Median        3Q       Max
-0.0098304 -0.0021966 -0.0000219  0.0013085  0.0126131

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0033529  0.0005896   5.687 9.72e-07 ***
data$V25    -0.0191828  0.0057158  -3.356  0.00164 **


Residual standard error: 0.003998 on 44 degrees of freedom
Multiple R-squared:  0.2038,    Adjusted R-squared:  0.1857
F-statistic: 11.26 on 1 and 44 DF,  p-value: 0.001637
================================================================
26. VRK3A

Residuals:
      Min        1Q    Median        3Q       Max
-0.0109938 -0.0018490 -0.0004393  0.0024643  0.0130403
```

```
Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0035761  0.0006332   5.648 1.11e-06 ***
data$V26    0.0117052  0.0053048   2.207   0.0326 *


Residual standard error: 0.004252 on 44 degrees of freedom
Multiple R-squared:  0.09963,   Adjusted R-squared:  0.07917
F-statistic: 4.869 on 1 and 44 DF,  p-value: 0.03261
=============================================================
27. YIF1A

Residuals:
      Min        1Q    Median        3Q       Max
-0.0093942 -0.0024695 -0.0008803  0.0027747  0.0139034

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0033035  0.0006377   5.181  5.3e-06 ***
data$V27    0.0103203  0.0055679   1.854   0.0705 .


Residual standard error: 0.004316 on 44 degrees of freedom
Multiple R-squared:  0.07243,   Adjusted R-squared:  0.05134
F-statistic: 3.436 on 1 and 44 DF,  p-value: 0.07052
```

Table 62: Test-statistic from selected features: mRNA versus rate of cognitive decline

(3c) Regression - rate of decline versus mRNA expression

```
=== Run information ===

Scheme:weka.classifiers.meta.CVParameterSelection -X 10 -S 1 -W weka.classifiers.functions.LinearRegression -- -S 0 -R 1.0E-8
Relation:     wml.rna_status-weka.filters.unsupervised.attribute.Reorder-..BestFirst -D 1 -N 5-
weka.filters.unsupervised.attribute.Remove-R1-2,8-9,14-15,24,27
Instances:    46
Attributes:   20
              CCR6_A
              CD81
              CHMP6
              CNOT2
              CTTN
              GNLY
              GPAA1
              HBA2_A
              HLAB
              PABPC1_A
              PPHLN1
              PRPF40A
              PSCD1_A
              REPS2
              RN7SL1
              RNF7
              SLC5A6
              TMEM59
              VRK3_A
              CDR-SOB
Test mode:evaluate on training data
```

```
=== Classifier model (full training set) ===

Cross-validated Parameter selection.
Classifier: weka.classifiers.functions.LinearRegression
Classifier Options: -S 0 -R 1.0E-8


Linear Regression Model

CDR-SOB =

      0.008  * CCR6_A +
     -0.0021 * CD81 +
      0.0023 * CTTN +
      0.0039 * GNLY +
     -0.0053 * GPAA1 +
     -0.0078 * HBA2_A +
      0.0041 * HLAB +
      0.0032 * PABPC1_A +
      0.0049 * PPHLN1 +
     -0.004  * PSCD1_A +
     -0.0055 * REPS2 +
      0.0047 * RN7SL1 +
      0.0038

Time taken to build model: 0 seconds

=== Evaluation on training set ===
=== Summary ===

Correlation coefficient                  0.9318
Mean absolute error                      0.0012
Root mean squared error                  0.0016
Relative absolute error                 38.3865 %
Root relative squared error             36.2901 %
Total Number of Instances               46

=== Evaluation on 10f cross-val training set ===

=== Cross-validation ===
=== Summary ===

Correlation coefficient                  0.7519
Mean absolute error                      0.0025
Root mean squared error                  0.0031
Relative absolute error                 74.9358 %
Root relative squared error             69.6695 %
Total Number of Instances               46


=== Evaluation on test set ===
=== Summary ===

Correlation coefficient                  0.3619
Mean absolute error                      0.0049
Root mean squared error                  0.0055
Relative absolute error                105.2673 %
Root relative squared error             89.7559 %
Total Number of Instances               16
```

Table 63: WEKA protocol rate of decline versus mRNA

(4a) Panel of proteins associated with the rate of cognitive decline identified with features selection

	Protein name	Chromo-somal location	Associated Gene Name [Alias] Gene ID	String-db/ Uniprot description
1	Interleukin 2 receptor	4q26-q27	IL2sRg IL2RB 3561	interleukin 2 receptor, beta; Receptor for interleukin-2. This beta subunit is involved in receptor mediated endocytosis and transduces the mitogenic signals of IL2
2	Erythropoietin receptor		EPOR 2057	Receptor for erythropoietin. Mediates erythropoietin- induced erythroblast proliferation and differentiation. Upon EPO stimulation, EPOR dimerizes triggering the JAK2/STAT5 signaling cascade. In some cell types, can also activate STAT1 and STAT3. May also activate the LYN tyrosine kinase
3	Hemopexin	11p15.5-p15.4	HPX 3263	Binds heme and transports it to the liver for breakdown and iron recovery, after which the free hemopexin returns to the circulation
4	Tumor Necrosis Factor Receptor Superfamily, Member 4	1p36	OX40 ligand TNFRSF4 7293	tumor necrosis factor receptor superfamily, member 4; Receptor for TNFSF4/OX40L/GP34
5	WNT inhibitory factor 1	12q14.2	WIF1 11197	WNT inhibitory factor 1; Binds to WNT proteins and inhibits their activities. May be involved in mesoderm segmentation (379 aa)
6	CXCL5/ chemokine (C-X-C motif) ligand 5	4q13.3	ENA78 6374	chemokine (C-X-C motif) ligand 5; Involved in neutrophil activation. In vitro, ENA-78(8- 78) and ENA-78(9-78) show a threefold higher chemotactic activity for neutrophil granulocytes (114 aa)
7	Sialic acid binding Ig-like lectin 9	19q13.3-q13.4	Siglec9 27180	sialic acid binding Ig-like lectin 9; Putative adhesion molecule that mediates sialic-acid dependent binding to cells. Preferentially binds to alpha-2,3- or alpha-2,6-linked sialic acid. The sialic acid recognition site may be masked by cis interactions with sialic acids on the same cell surface (463 aa)
8	Interleukin 19	1q32.2	IL19 29949	May play some important roles in inflammatory responses. Up-regulates IL-6 and TNF-alpha and induces apoptosis (By similarity) (215 aa)
9	HMGCR/ HMG-CoA reductase	5q13.3-q14	CRIS3 INSIG2 3156	insulin induced gene 2; Mediates feedback control of cholesterol synthesis by controlling SCAP and HMGCR. Functions by blocking the processing of sterol regulatory element-binding proteins (SREBPs). Capable of retaining the SCAP-SREBF2 complex in the ER thus preventing it from escorting SREBPs to the Golgi. Seems to regulate the ubiquitin-mediated proteasomal degradation of HMGCR (225 aa)
10	Tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor	2q32	TFPI 7035	Inhibits factor X (X(a)) directly and, in a Xa-dependent way, inhibits VIIa/tissue factor activity, presumably by forming a quaternary Xa/LACI/VIIa/TF complex. It possesses an

	Protein name	Chromo-somal location	Associated Gene Name [Alias] Gene ID	String-db/ Uniprot description
				antithrombotic action and also the ability to associate with lipoproteins in plasma (304 aa)
11	Arnosine dipeptidase 1 (metallopeptidase M20 family	18q22.3	CNDP1 84735	carnosine dipeptidase 1 (metallopeptidase M20 family) (507 aa)
12	Coagulation factor II	11p11.2	Thrombin 14061	coagulation factor II (thrombin); Thrombin, which cleaves bonds after Arg and Lys, converts fibrinogen to fibrin and activates factors V, VII, VIII, XIII, and, in complex with thrombomodulin, protein C. Functions in blood homeostasis, inflammation and wound healing (622 aa)
13	Ectonucleoside triphosphate diphosphohydrolase 3	3p21.3	ENTP3 ENTPD3 956	ectonucleoside triphosphate diphosphohydrolase 3; Has a threefold preference for the hydrolysis of ATP over ADP (529 aa)
14	Interleukin 6	7p21-p15	IL6 3569	interleukin 6 (interferon, beta 2); Cytokine with a wide variety of biological functions. It is a potent inducer of the acute phase response. Plays an essential role in the final differentiation of B-cells into Ig- secreting cells Involved in lymphocyte and monocyte differentiation. It induces myeloma and plasmacytoma growth and induces nerve cells differentiation Acts on B-cells, T-cells, hepatocytes, hematopoeitic progenitor cells and cells of the CNS. Also acts as a myokine. It is discharged into the bloodstream after muscle contraction and acts to increase the breakdown of fats and [...] (212 aa)
15	Bone marrow stromal cell antigen 1	4p15	BST1 683	Synthesizes cyclic ADP-ribose, a second messenger that elicits calcium release from intracellular stores. May be involved in pre-B-cell growth (318 aa)
16	Chromobox homolog 5	12q13.13	CBX5 23468	chromobox homolog 5 (HP1 alpha homolog, Drosophila); Component of heterochromatin. Recognizes and binds histone H3 tails methylated at 'Lys-9', leading to epigenetic repression. Can interact with lamin B receptor (LBR). This interaction can contribute to the association of the heterochromatin with the inner nuclear membrane. Involved in the formation of functional kinetochore through interaction with MIS12 complex proteins (191 aa)

Table 64: Panel of proteins associated with the rate of cognitive decline identified with features selection

(4b) Summary of test statistics from feature selection proteins versus rate of cognitive decline

```
1. IL_2_sRg

Residuals:
      Min        1Q    Median        3Q       Max
-0.0057880 -0.0021092 -0.0003732  0.0020204  0.0080228

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0039280  0.0004692   8.372 9.97e-11 ***
data$V1     0.0141571  0.0051915   2.727  0.00908 **


Residual standard error: 0.003217 on 45 degrees of freedom
Multiple R-squared:  0.1418,    Adjusted R-squared:  0.1227
F-statistic: 7.436 on 1 and 45 DF,  p-value: 0.009079
============================================================
2. EPO_R

Residuals:
      Min        1Q    Median        3Q       Max
-0.0066778 -0.0022814 -0.0005287  0.0017395  0.0079883

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0039786  0.0004841   8.219 1.66e-10 ***
data$V2     0.0066348  0.0031731   2.091   0.0422 *


Residual standard error: 0.003315 on 45 degrees of freedom
Multiple R-squared:  0.08855,   Adjusted R-squared:  0.0683
F-statistic: 4.372 on 1 and 45 DF,  p-value: 0.04221
============================================================
3. Hemopexin

Residuals:
      Min        1Q    Median        3Q       Max
-0.0053710 -0.0025686 -0.0004959  0.0020547  0.0077094

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0038195  0.0004732   8.071 2.72e-10 ***
data$V3     0.0015471  0.0005867   2.637   0.0114 *


Residual standard error: 0.003231 on 45 degrees of freedom
Multiple R-squared:  0.1338,    Adjusted R-squared:  0.1146
F-statistic: 6.953 on 1 and 45 DF,  p-value: 0.01144
============================================================
4. OX40 Ligand

Residuals:
      Min        1Q    Median        3Q       Max
-0.0071079 -0.0024594 -0.0007552  0.0027646  0.0097087

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0036875  0.0005174   7.127  6.6e-09 ***
data$V4     -0.0049997  0.0032046  -1.560    0.126
```

```
Residual standard error: 0.003382 on 45 degrees of freedom
Multiple R-squared:  0.05131,   Adjusted R-squared:  0.03023
F-statistic: 2.434 on 1 and 45 DF,  p-value: 0.1257
================================================================
5. WIF1

Residuals:
      Min        1Q    Median        3Q       Max
-0.0072272 -0.0021012 -0.0001657  0.0022372  0.0088344

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0041333  0.0005053   8.179 1.89e-10 ***
data$V5     -0.0080178  0.0047306  -1.695    0.097 .


Residual standard error: 0.003366 on 45 degrees of freedom
Multiple R-squared:  0.06001,   Adjusted R-squared:  0.03912
F-statistic: 2.873 on 1 and 45 DF,  p-value: 0.09701
================================================================
6. ENA78

Residuals:
      Min        1Q    Median        3Q       Max
-0.0065512 -0.0018840 -0.0004982  0.0020962  0.0085624

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0038649  0.0004606   8.392 9.34e-11 ***
data$V6     0.0110981  0.0035942   3.088  0.00345 **


Residual standard error: 0.003154 on 45 degrees of freedom
Multiple R-squared:  0.1748,    Adjusted R-squared:  0.1565
F-statistic: 9.535 on 1 and 45 DF,  p-value: 0.003448
================================================================
7. Siglec9

Residuals:
      Min        1Q    Median        3Q       Max
-0.0062476 -0.0022761  0.0002229  0.0018106  0.0066462

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0041322  0.0004787   8.631 4.23e-11 ***
data$V7     -0.0013841  0.0005338  -2.593   0.0128 *


Residual standard error: 0.003239 on 45 degrees of freedom
Multiple R-squared:   0.13,       Adjusted R-squared:  0.1107
F-statistic: 6.723 on 1 and 45 DF,  p-value: 0.01279
================================================================
8. IL19

Residuals:
      Min        1Q    Median        3Q       Max
-0.0066678 -0.0024809 -0.0002979  0.0021453  0.0075960

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0038616  0.0004959   7.786 7.07e-10 ***
data$V8     0.0054714  0.0035872   1.525    0.134


Residual standard error: 0.003386 on 45 degrees of freedom
```

```
Multiple R-squared:  0.04915,   Adjusted R-squared:  0.02802
F-statistic: 2.326 on 1 and 45 DF,  p-value: 0.1342
============================================================
9. CRIS3

Residuals:
      Min        1Q    Median        3Q       Max
-0.0062711 -0.0024487  0.0003294  0.0018387  0.0082103

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0032784  0.0005452   6.013 2.98e-07 ***
data$V9     -0.0211286  0.0086244  -2.450   0.0182 *


Residual standard error: 0.003261 on 45 degrees of freedom
Multiple R-squared:  0.1177,    Adjusted R-squared:  0.09807
F-statistic: 6.002 on 1 and 45 DF,  p-value: 0.01825
============================================================
10. TFPI

Residuals:
     Min       1Q   Median       3Q      Max
-0.006717 -0.001559 -0.000721  0.002033  0.007625

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0039230  0.0004452   8.812 2.34e-11 ***
data$V10    0.0051422  0.0014132   3.639 0.000703 ***


Residual standard error: 0.003052 on 45 degrees of freedom
Multiple R-squared:  0.2273,    Adjusted R-squared:  0.2102
F-statistic: 13.24 on 1 and 45 DF,  p-value: 0.000703
============================================================
11. CNDP1

Residuals:
     Min       1Q   Median       3Q      Max
-0.007250 -0.002364 -0.001181  0.002275  0.008311

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0040304  0.0005006   8.051  2.9e-10 ***
data$V11    0.0016314  0.0011557   1.412    0.165


Residual standard error: 0.003398 on 45 degrees of freedom
Multiple R-squared:  0.0424,    Adjusted R-squared:  0.02112
F-statistic: 1.993 on 1 and 45 DF,  p-value: 0.165
============================================================
12. Thrombin

Residuals:
      Min        1Q    Median        3Q       Max
-0.0073623 -0.0018696 -0.0001726  0.0018552  0.0075946

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0041584  0.0004656   8.932 1.58e-11 ***
data$V12    0.0124501  0.0040164   3.100  0.00334 **


Residual standard error: 0.003152 on 45 degrees of freedom
Multiple R-squared:  0.176,     Adjusted R-squared:  0.1576
F-statistic: 9.609 on 1 and 45 DF,  p-value: 0.003335
============================================================
```

```
13. ENTP3

Residuals:
     Min       1Q    Median       3Q      Max
-0.006799 -0.001748 -0.000639  0.002515  0.007708

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0036672  0.0004963   7.389 2.71e-09 ***
data$V13    0.0100875  0.0046085   2.189   0.0338 *


Residual standard error: 0.003301 on 45 degrees of freedom
Multiple R-squared:  0.09623,   Adjusted R-squared:  0.07614
F-statistic: 4.791 on 1 and 45 DF,  p-value: 0.03383
============================================================
14. IL6

Residuals:
      Min        1Q     Median        3Q       Max
-0.0066311 -0.0020086 -0.0005082  0.0020258  0.0079995

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0038437  0.0004834   7.951 4.06e-10 ***
data$V14    -0.0165336  0.0075982  -2.176   0.0348 *


Residual standard error: 0.003303 on 45 degrees of freedom
Multiple R-squared:  0.0952,    Adjusted R-squared:  0.0751
F-statistic: 4.735 on 1 and 45 DF,  p-value: 0.03485
============================================================
15. BST1

Residuals:
      Min        1Q     Median        3Q       Max
-0.0055037 -0.0022141 -0.0006536  0.0018923  0.0064133

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)  0.0040280  0.0004661   8.642 4.09e-11 ***
data$V15    -0.0057518  0.0019836  -2.900  0.00576 **


Residual standard error: 0.003187 on 45 degrees of freedom
Multiple R-squared:  0.1574,    Adjusted R-squared:  0.1387
F-statistic: 8.408 on 1 and 45 DF,  p-value: 0.005757
============================================================
16. CBX5
Residuals:
     Min       1Q    Median       3Q      Max
-0.006373 -0.002206 -0.000886  0.001941  0.008807

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) 0.0037612  0.0004951   7.598 1.34e-09 ***
data$V16    0.0078821  0.0041203   1.913   0.0621 .


Residual standard error: 0.003339 on 45 degrees of freedom
Multiple R-squared:  0.07521,   Adjusted R-squared:  0.05466
F-statistic:  3.66 on 1 and 45 DF,  p-value: 0.06212
```

Table 65: Test-statistic from selected features: proteins versus rate of cognitive decline

(4c) Regression - rate of decline versus protein concentration

```
=== Run information ===

Scheme:weka.classifiers.meta.CVParameterSelection -P "R -5.99999999 5.0E-4 5.00000001" -P "C 0.0 1.0 1.0" -X 10 -S 1 -W
weka.classifiers.functions.LinearRegression -- -S 0 -R 1.0E-8
Relation:     wml.rna_status-weka.filters.unsupervised.attribute.Reorder-R2,3,..,1017,1-
weka.filters.supervised.attribute.AttributeSelection-Eweka.attributeSelection.CfsSubsetEval-Sweka.attributeSelection.BestFirst -D
1 -N 5
Instances:    47
Attributes:   17
              IL_2_sRg
              EPO_R
              Hemopexin
              OX40_Ligand
              WIF_1
              ENA_78
              Siglec_9
              IL_19
              CRIS3
              TFPI
              CNDP1
              Thrombin
              ENTP3
              IL_6
              BST1
              CBX5
              CDR-SOB
Test mode:evaluate on training data

=== Classifier model (full training set) ===

Cross-validated Parameter selection.
Classifier: weka.classifiers.functions.LinearRegression
Cross-validation Parameter: '-R' ranged from -5.99999999 to 5.0E-4 with 5.00000001 steps
Cross-validation Parameter: '-C' ranged from 0.0 to 1.0 with 1.0 steps
Classifier Options: -R 4.999849987497029E-4 -C 0 -S 0


Linear Regression Model

CDR-SOB =

      0.0057 * EPO_R +
     -0.0037 * OX40_Ligand +
      0.0097 * ENA_78 +
      0.0064 * IL_19 +
     -0.0166 * CRIS3 +
      0.0033 * TFPI +
     -0.0098 * IL_6 +
     -0.0031 * BST1 +
      0.0031

Time taken to build model: 0.02 seconds

=== Evaluation on training set ===
=== Summary ===

Correlation coefficient                  0.8383
Mean absolute error                      0.0013
Root mean squared error                  0.0019
Relative absolute error                 48.0469 %
Root relative squared error             54.524  %
Total Number of Instances               47
```

=== Cross-validation ===
=== Summary ===

Correlation coefficient	0.5946
Mean absolute error	0.0021
Root mean squared error	0.003
Relative absolute error	77.4313 %
Root relative squared error	87.1138 %
Total Number of Instances	47

=== Re-evaluation on test set ===

User supplied test set
Relation: wml.rna_status-weka.filters.unsupervised.attribute.Reorder-R2...1016
Instances: unknown (yet). Reading incrementally
Attributes: 17

=== Summary ===

Correlation coefficient	-0.002
Mean absolute error	0.0053
Root mean squared error	0.0069
Total Number of Instances	16

Table 66: WEKA protocol - rate of decline versus proteins

C. Protein sequence information

mRNA/WMH

ALAS2, ARFGAP1, CCDC90A, CREB5, EOMES, FAU, FBXW4, LILRA5, HS.127310, NAG18, NCAPD2, PNKD, RRAGC, SHARPIN

>**ALAS2**
MVTAAMLLQCCPVLARGPTSLLGKVVKTHQFLFGIGRCPILATQGPNCSQIHLKATKAGGDSPSWAKGHCPFMLSELQDG
KSKIVQKAAPEVQEDVKAFKTDLPSSLVSVSLRKPFSGPQEQEQISGKVTHLIQNNMPGNYVFSYDQFFRDKIMEKKQDH
TYRVFKTVNRWADAYPFAQHFSEASVASKDVSVWCSNDYLGMSRHPQVLQATQETLQRHGAGAGGTRNISGTSKFHVELE
QELAELHQKDSALLFSSCFVANDSTLFTLAKILPGCEIYSDAGNHASMIQGIRNSGAAKFVFRHNDPDHLKKLLEKSNPK
IPKIVAFETVHSMDGAICPLEELCDVSHQYGALTFVDEVHAVGLYGSRGAGIGERDGIMHKIDIISGTLGKAFGCVGGYI
ASTRDLVDMVRSYAAGFIFTTSLPPMVLSGALESVRLLKGEEGQALRRAHQRNVKHMRQLLMDRGLPVIPCPSHIIPIRV
GNAALNSKLCDLLLSKHGIYVQAINYPTVPRGEELLRLAPSPHHSPQMMEDFVEKLLLAWTAVGLPLQDVSVAACNFCRR
PVHFELMSEWERSYFGNMGPQYVTTYA
>**ARFGAP1**
MASPRTRKVLKEVRVQDENNVCFECGAFNPQWVSVTYGIWICLECSGRHRGLGVHLSFVRSVTMDKWKDIELEKMKAGG
N
AKFREFLESQEDYDPCWSLQEKYNSRAAALFRDKVVALAEGREWSLESSPAQNWTPPQPRTLPSMVHRVSGQPQSVTASS

DKAFEDWLNDDLGSYQGAQGNRYVGFGNTPPPQKKEDDFLNNAMSSLYSGWSSFTTGASRFASAAKEGATKFGSQASQKF
WGHKQQPEPASELGHSLNENVLKPAQEKVKEGKIFDDVSSGVSQLASKGVGSKGWRDVTTFFSGKAEGPLDSPSEGHSYQ
NSGLDHFQNSNIDQSFWETFGSAEPTKTRKSPSSDSWTCADTSTERRSSDSWEVWGSASTNRNSNSDGGEGGEGTKKAVP
PAVPTDDGWDNQNW

>**CCDC90A**

MDCGSVGGQRTQRLPGRQRLLFLPVGLSGRPGGSETSARRCLSALSDGLGALRPRAPAARGGVSRASPLLLLLLVPSPRL
AAAAPRRQLGDWERSRLGYAAPPAGRSSAWRCSPGVAAAAGALPQYHGPAPALVSCRRELSLSAGSLQLERKRRDFTSSG
SRKLYFDTHALVCLLEDNGFATQQAEIIVSALVKILEANMDIVYKDMVTKMQQEITFQQVMSQIANVKKDMIILEKSEFS
ALRAENEKIKLELHQLKQQVMDEVIKVRTDTKLDFNLEKSRVKELYSLNEKKLLELRTEIVALHAQQDRALTQTDRKIET
EVAGLKTMLESHKLDNIKYLAGSIFTCLTVALGFYRLWI

>**CREB5**

MIYEESKMNLEQERPFVCSAPGCSQRFPTEDHLMIHRHKHEMTLKFPSIKTDNMLSDQTPTPTRFLKNCEEVGLFSELDC
SLEHEFRKAQEEEESSKRNISMHNAVGGAMTGPGTHQLSSARLPNHDTNVVIQQAMPSPQSSSVITQAPSTNRQIGPVPGS
LSSLLHLHNRQRQPMPASMPGTLPNPTMPGSSAVLMPMERQMSVNSSIMGMQGPNLSNPCASPQVQPMHSEAKMRLKAA
L
THHPAAMSNGNMNTMGHMMEMMGSRQDQTPHHHMHSHPHQHQTLPPHHPYPHQHQHPAHHPHPQPHHQQNHPHHHSH
SHL
HAHPAHHQTSPHPPLHTGNQAQVSPATQQMQPTQTIQPPQPTGGRRRRVVDEDPDERRRKFLERNRAAATRCRQKRKVWV
MSLEKKAEELTQTNMQLQNEVSMLKNEVAQLKQLLLTHKDCPITAMQKESQGYLSPESSPPASPVPACSQQQVIQHNTIT
TSSSVSEVVGSSTLSQLTTHRTDLNPIL

>**EOMES**

MQLGEQLLVSSVNLPGAHFYPLESARGGSGGSAGHLPSAAPSPQKLDLDKASKKFSGSLSCEAVSGEPAAASAGAPAAML
SDTDAGDAFASAAAVAKPGPPDGRKGSPCGEEELPSAAAAAAAAAAAAAATARYSMDSLSSERYYLQSPGPQGSELAAPC
SLFPYQAAAGAPHGPVYPAPNGARYPYGSMLPPGGFPAAVCPPGRAQFGPGAGAGSGAGGSSGGGGGPGTYQYSQGAPLY
GPYPGAAAAGSCGGLGGLGVPGSGFRAHVYLCNRPLWLKFHRHQTEMIITKQGRRMFPFLSFNINGLNPTAHYNVFVEVV
LADPNHWRFQGGKWVTCGKADNNMQGNKMYVHPESPNTGSHWMRQEISFGKLKLTNNKGANNNNTQMIVLQSLHKYQ
PRL
HIVEVTEDGVEDLNEPSKTQTFTFSETQFIAVTAYQNTDITQLKIDHNPFAKGFRDNYDSSHQIVPGGRYGVQSFFPEPF
VNTLPQARYYNGERTVPQTNGLLSPQQSEEVANPPQRWLVTPVQQPGTNKLDISSYESEYTSSTLLPYGIKSLPLQTSHA
LGYYPDPTFPAMAGWGGRGSYQRKMAAGLPWTSRTSPTVFSEDQLSKEKVKEEIGSSWIETPPSIKSLDSNDSGVYTSAC
KRRRLSPSNSSNENSPSIKCEDINAEEYSKDTSKGMGGYYAFYTTP

>**FAU**

MQLFVRAQELHTFEVTGQETVAQIKAHVASLEGIAPEDQVVLLAGAPLEDEATLGQCGVEALTTLEVAGRMLGGKVHGSL
ARAGKVRGQTPKVAKQEKKKKKTGRAKRRMQYNRRFVNVVPTFGKKKGPNANS

>**FBXW4**

MAAAAGEEEEEEEAARESAARPAAGPALWRLPEELLLLICSYLDMRALGRLAQVCRWLRRFTSCDLLWRRIARASLNSGF
TRLGTDLMTSVPVKERVKVSQNWRLGRCREGILLKWRCSQMPWMQLEDDSLYISQANFILAYQFRPDGASLNRRPLGVFA
GHDEDVCHFVLANSHIVSAGGDGKIGIHKIHSTFTVKYSAHEQEVNCVDCKGGIIVSGSRDRTAKVWPLASGRLGQCLHT
IQTEDRVWSIAISPLLSSFVTGTACCGHFSPLRIWDLNSGQLMTHLGSDFPPGAGVLDVMYESPFTLLSCGYDTYVRYWD
LRTSVRKCVMEWEEPHDSTLYCLQTDGNHLLATGSSYYGVVRLWDRRQRACLHAFPLTSTPLSSPVYCLRLTTKHLYAAL
SYNLHVLDFQNP

>LILRA5

MAPWSHPSAQLQPVGGDAVSPALMVLLCLGLSLGPRTHVQAGNLSKATLWAEPGSVISRGNSVTIRCQGTLEAQEYRLVK
EGSPEPWDTQNPLEPKNKARFSIPSMTEHHAGRYRCYYYSPAGWSEPSDPLELVVTGFYNKPTLSALPSPVVTSGENVTL
QCGSRLRFDRFILTEEGDHKLSWTLDSQLTPSGQFQALFPVGPVTPSHRWMLRCYGSRRHILQVWSEPSDLLEIPVSGAA

DNLSPSQNKSDSGTASHLQDYAVENLIRMGMAGLILVVLGILIFQDWHSQRSPQAAAGR

>**HS.127310**

MAGSGCAWGAEPPRFLEAFGRLWQVQSRLGSGSSASVYRVRCCGNPGSPPGALKQFLPPGTTGAAASAAEYGFRKERAAL
EQLQGHRNIVTLYGVFTIHFSPNVPSRCLLLELLDVSVSELLLYSSHQGCSMWMIQHCARDVLEALAFLHHEGYVHADLK
PRNILWSAENECFKLIDFGLSFKEGNQDVKYIQTDGYRAPEAELQNCLAQAGLQSDTECTSAVDLWSLGIILLEMFSGMK
LKHTVRSQEWKANSSAIIDHIFASKAVVNAAIPAYHLRDLIKSMLHDDPSRRIPAEMALCSPFFSIPFAPHIEDLVMLPT
PVLRLLNVLDDDYLENEEEYEDVVEDVKEECQKYGPVVSLLVPKENPGRGQVFVEYANAGDSKAAQKLLTGRMFDGKFV
V

ATFYPLSAYKRGYLYQTLL

>**NAG18**

MDGNGGHYLSEISQEHKHAHSHSYVEALKKKKKAGCGGSRLQSQHPGRLRRVDHLRSGVQ
DQPDQHGETLSPPKTQN

>**NCAPD2**

MAPQMYEFHLPLSPEELLKSGGVNQYVVQEVLSIKHLPPQLRAFQAAFRAQGPLAMLQHFDTIYSILHHFRSIDPGLKED
TLQFLIKVVSRHSQELPAILDDTTLSGSDRNAHLNALKMNCYALIRLLESFETMASQTNLVDLDLGGKGKKARTKAAHGF
DWEEERQPILQLLTQLLQLDIRHLWNHSIIEEEFVSLVTGCCYRLLENPTINHQKNRPTREAITHLLGVALTRYNHMLSA
TVKIIQMLQHFEHLAPVLVAAVSLWATDYGMKSIVGEIVREIGQKCPQELSRDPSGTKGFAAFLTELAERVPAILMSSMC
ILLDHLDGENYMMRNAVLAAMAEMVLQVLSGDQLEAAARDTRDQFLDTLQAHGHDVNSFVRSRVLQLFTRIVQQKALPL
T

RFQAVVALAVGRLADKSVLVCKNAIQLLASFLANNPFSCKLSDADLAGPLQKETQKLQEMRAQRRTAAASAVLDPEEEWE
AMLPELKSTLQQLLQLPQGEEEIPEQIANTETTEDVKGRIYQLLAKASYKKAIILTREATGHFQESEPFSHIDPEESEET
RLLNILGLIFKGPAASTQEKNPRESTGNMVTGQTVCKNKPNMSDPEESRGNDELVKQEMLVQYLQDAYSFSRKITEAIGI
ISKMMYENTTTVVQEVIEFFVMVFQFGVPQALFGVRRMLPLIWSKEPGVREAVLNAYRQLYLNPKGDSARAKAQALIQNL
SLLLVDASVGTIQCLEEILCEFVQKDELKPAVTQLLWERATEKVACCPLERCSSVMLLGMMARGKPEIVGSNLDTLVSIG
LDEKFPQDYRLAQQVCHAIANISDRRKPSLGKRHPPFRLPQEHRLFERLRETVTKGFVHPDPLWIPFKEVAVTLIYQLAE
GPEVICAQILQGCAKQALEKLEEKRTSQEDPKESPAMLPTFLLMNLLSLAGDVALQQLVHLEQAVSGELCRRRVLREEQE
HKTKDPKEKNTSSETTMEEELGLVGATADDTEAELIRGICEMELLDGKQTLAAFVPLLLKVCNNPGLYSNPDLSAAASLA
LGKFCMISATFCDSQLRLLFTMLEKSPLPIVRSNLMVATGDLAIRFPNLVDPWTPHLYARLRDPAQQVRKTAGLVMTHLI
LKDMVKVKGQVSEMAVLLIDPEPQIAALAKNFFNELSHKGNAIYNLLPDIISRLSDPELGVEEEPFHTIMKQLLSYITKD
KQTESLVEKLCQRFRTSRTERQQRDLAYCVSQLPLTERGLRKMLDNFDCFGDKLSDESIFSAFLSVVGKLRRGAKPEGKA
IIDEFEQKLRACHTRGLDGIKELEIGQAGSQRAPSAKKPSTGSRYQPLASTASDNDFVTPEPRRTTRRHPNTQQRASKKK
PKVVFSSDESSEEDLSAEMTEDETPKKTTPILRASARRHRS

>**PNKD**

MAAVVAATALKGRGARNARVLRGILAGATANKASHNRTRALQSHSSPEGKEEPEPLSPELEYIPRKRGKNPMKAVGLAWY
SLYTRTWLGYLFYRQQLRRARNRYPKGHSKTQPRLFNGVKVLPIPVLSDNYSYLIIDTQAQLAVAVDPSDPRAVQASIEK
EGVTLVAILCTHKHWDHSGGNRDLSRRHRDCRVYGSPQDGIPYLTHPLCHQDVVSVGRLQIRALATPGHTQGHLVYLLDG
EPYKGPSCLFSGDLLFLSGCGRTFEGNAETMLSSLDTVLGLGDDTLLWPGHEYAEENLGFAGVVEPENLARERKMQWVQR
QRLERKGTCPSTLGEERSYNPFLRTHCLALQEALGPGPGPTGDDDYSRAQLLEELRRLKDMHKSK

>**RRAGC**

MSLQYGAEETPLAGSYGAADSFPKDFGYGVEEEEEEAAAAGGGVGAGAGGGCGPGGADSSKPRILLMGLRRSGKSSIQKV
VFHKMSPNETLFLESTNKIYKDDISNSSFVNFQIWDFPGQMDFFDPTFDYEMIFRGTGALIYVIDAQDDYMEALTRLHIT
VSKAYKVNPDMNFEVFIHKVDGLSDDHKIETQRDIHQRANDDLADAGLEKLHLSFYLTSIYDHSIFEAFSKVVQKLIPQL
PTLENLLNIFISNSGIEKAFLFDVVSKIYIATDSSPVDMQSYELCCDMIDVVIDVSCIYGLKEDGSGSAYDKESMAIIKL
NNTTVLYLKEVTKFLALVCILREESFERKGLIDYNFHCFRKAIHEVFEVGVTSHRSCGHQTSASSLKALTHNGTPRNAI

>**SHARPIN**

MAPPAGGAAAAASDLGSAAVLLAVHAAVRPLGAGPDAEAQLRRLQLSADPERPGRFRLELLGAGPGAVNLEWPLESVSYT
IRGPTQHELQPPPGGPGTLSLHFLNPQEAQRWAVLVRGATVEGQNGSKSNSPPALGPEACPVSLPSPPEASTLKGPPPEA
DLPRSPGNLTEREELAGSLARAIAGGDEKGAAQVAAVLAQHRVALSVQLQEACFPPGPIRLQVTLEDAASAASAASSAHV
ALQVHPHCTVAALQEQVFSELGFPPAVQRWVIGRCLCVPERSLASYGVRQDGDPAFLYLLSAPREAPATGPSPQHPQKMD
GELGRLFPPSLGLPPGPQPAASSLPSPLQPSWSCPSCTFINAPDRPGCEMCSTQRPCTWDPLAAAST

Proteins/WMH

CXCL16 = CXCL16 soluable**, GDF11, ALPL =**Alkaline phospatase bone**, DOK7 =** lgM**, SPOK1 =** Testican **, CHEK2 =**CHK2**, BCL2**, **SERPINA4 =** Kallistatin**, VIP =**Vasoactive intestine Peptide**, CNDP1, CTSH=**Cathepsin H, **MBD4, LCMT1, SAA1=SAA, CYTH2=**PH**, FAM107B, F5, C1QBP, ESD=**Esterase D**, GPC5, STK16, MME=MMP12**

>**CXCL16**
MSGSQSEVAPSPQSPRSPEMGRDLRPGSRVLLLLLLLLLVYLTQPGNGNEGSVTGSCYCGKRISSDSPPSVQFMNRLRKH
LRAYHRCLYYTRFQLLSWSVCGGNKDPWVQELMSCLDLKECGHAYSGIVAHQKHLLPTSPPISQASEGASSDIHTPAQML
LSTLQSTQRPTLPVGSLSSDKELTRPNETTIHTAGHSLAAGPEAGENQKQPEKNAGPTARTSATVPVLCLLAIIFILTAA
LSYVLCKRRRGQSPQSSPDLPVHYIPVAPDSNT
>GDF11
MVLAAPLLLGFLLLALELRPRGEAAEGPAAAAAAAAAAAAAGVGGERSSRPAPSVAPEPDGCPVCVWRQHSRELRLESIK
SQILSKLRLKEAPNISREVVKQLLPKAPPLQQILDLHDFQGDALQPEDFLEEDEYHATTETVISMAQETDPAVQTDGSPL
CCHFHFSPKVMFTKVLKAQLWVYLRPVPRPATVYLQILRLKPLTGEGTAGGGGGGRRHIRIRSLKIELHSRSGHWQSIDF
KQVLHSWFRQPQSNWGIEINAFDPSGTDLAVTSLGPGAEGLHPFMELRVLENTKRSRRNLGLDCDEHSSESRCCRYPLTV
DFEAFGWDWIIAPKRYKANYCSGQCEYMFMQKYPHTHLVQQANPRGSAGPCCTPTKMSPINMLYFNDKQQIIYGKIPGMV
VDRCGCS
>**ALPL**
MISPFLVLAIGTCLTNSLVPEKEKDPKYWRDQAQETLKYALELQKLNTNVAKNVIMFLGDGMGVSTVTAARILKGQLHHN
PGEETRLEMDKFPFVALSKTYNTNAQVPDSAGTATAYLCGVKANEGTVGVSAATERSRCNTTQGNEVTSILRWAKDAGKS
VGIVTTTRVNHATPSAAYAHSADRDWYSDNEMPPEALSQGCKDIAYQLMHNIRDIDVIMGGGRKYMYPKNKTDVEYESDE
KARGTRLDGLDLVDTWKSFKPRYKHSHFIWNRTELLTLDPHNVDYLLGLFEPGDMQYELNRNNVTDPSLSEMVVVAIQIL
RKNPKGFFLLVEGGRIDHGHHEGKAKQALHEAVEMDRAIGQAGSLTSSEDTLTVVTADHSHVFTFGGYTPRGNSIFGLAP
MLSDTDKKPFTAILYGNGPGYKVVGGERENVSMVDYAHNNYQAQSAVPLRHETHGGEDVAVFSKGPMAHLLHGVHEQNY
V
PHVMAYAACIGANLGHCAPASSAGSLAAGPLLLALALYPLSVLF
>**DOK7**
MTEAALVEGQVKLRDGKKWKSRWLVLRKPSPVADCLLMLVYKDKSERIKGLRERSSLTLEDICGLEPGLPYEGLVHTLAI
VCLSQAIMLGFDSHEAMCAWDARIRYALGEVHRFHVTVAPGTKLESGPATLHLCNDVLVLARDIPPAVTGQWKLSDLRRY
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>**SPOK1**
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>**CHEK2**

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>**BCL2**

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>SERPINA4

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>VIP

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>CNDP1

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>CTSH

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>MBD4

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>LCMT1

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>SAA1

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>CYTH2

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>FAM107B

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>F5

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mRNA/cognitive decline

CCR6=CCR6A, CD81, CTTN, GNLY, GPAA1, HBA2 =HBA2A, HLAB, PABC1=PABPC1A, PPHLN1, CYTH1=PSCD1A, REPS2

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>HBA2

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>HLAB

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>PABC1

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>PPHLN1

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>CYTH1

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PNCFELYIPDNKDQVIKACKTEADGRVVEGNHTVYRISAPTPEEKEEWIKCIKAAISRDPFYEMLAARKKKVSSTKRH

>REPS2

MEAAAAAAAAAAAAAAAAGGGCGSGPPPLLLSEGEQQCYSELFARCAGAAGGGPGSGPPEAARVAPGTATAAAGPVADLFR
ASQLPAETLHQITELCGAKRVGYFGPTQFYIALKLIAAAQSGLPVRIESIKCELPLPRFMMSKNDGEIRFGNPAELHGTK
VQIPYLTTEKNSFKRMDDEDKQQETQSPTMSPLASPPSSPPHYQRVPLSHGYSKLRSSAEQMHPAPYEARQPLVQPEGSS
SGGPGTKPLRHQASLIRSFSVERELQDNSSYPDEPWRITEEQREYYVNQFRSLQPDPSSFISGSVAKNFFTKSKLSIPEL
SYIWELSDADCDGALTLPEFCAAFHLIVARKNGYPLPEGLPPTLQPEYLQAAFPKPKWDCQLFDSYSESLPANQQPRDLN

RMEKTSVKDMADLPVPNQDVTSDDKQALKSTINEALPKDVSEDPATPKDSNSLKARPRSRSYSSTSIEEAMKRGEDPPTP
PPRPQKTHSRASSLDLNKVFQPSVPATKSGLLPPPPALPPRPCPSQSEQVSEAELLPQLSRAPSQAAESSPAKKDVLYSQ
PPSKPIRRKFRPENQATENQEPSTAASGPASAATMKPHPTVQKQSSKQKKAIQTAIRKNKEANAVLARLNSELQQQLKEV
HQERIALENQLEQLRPVTVL

Proteins/cognitive decline

EPOR, OX40 ligand =TNFRSF4, ENA78 =CXCL5, IL19, CRIS3=HMGCR, TFPI, IL6, BST1

>EPOR
MDHLGASLWPQVGSLCLLLAGAAWAPPPNLPDPKFESKAALLAARGPEELLCFTERLEDLVCFWEEAASAGVGPGNYSFS
YQLEDEPWKLCRLHQAPTARGAVRFWCSLPTADTSSFVPLELRVTAASGAPRYHRVIHINEVVLLDAPVGLVARLADESG
HVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPV
SLLTPSDLDPLILTLSLILVVILVLLTVLALLSHRRALKQKIWPGIPSPESEFEGLFTTHKGNFQLWLYQNDGCLWWSPC
TPFTEDPPASLEVLSERCWGTMQAVEPGTDDEGPLLEPVGSEHAQDTYLVLDKWLLPRNPPSEDLPGPGGSVDIVAMDEG
SEASSCSSALASKPSPEGASAASFEYTILDPSSQLLRPWTLCPELPPTPPHLKYLYLVVSDSGISTDYSSGDSQGAQGGL
SDGPYSNPYENSLIPAAEPLPPSYVACS
>TNFRSF4
MCVGARRLGRGPCAALLLLGLGLSTVTGLHCVGDTYPSNDRCCHECRPGNGMVSRCSRSQNTVCRPCGPGFYNDVVSSKP
CKPCTWCNLRSGSERKQLCTATQDTVCRCRAGTQPLDSYKPGVDCAPCPPGHFSPGDNQACKPWTNCTLAGKHTLQPASN
SSDAICEDRDPPATQPQETQGPPARPITVQPTEAWPRTSQGPSTRPVEVPGGRAVAAILGLGLVLGLLGPLAILLALYLL
RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI
>CXCL5
MSLLSSRAARVPGPSSSLCALLVLLLLLTQPGPIASAGPAAAVLRELRCVCLQTTQGVHPKMISNLQVFAIGPQCSKVEV
VASLKNGKEICLDPEAPFLKKVIQKILDGGNKEN
>IL19
MCTEGAFPHRSACSLPLTHVHTHIHVCVPVLWGSVPRGMKLQCVSLWLLGTILILCSVDNHGLRRCLISTDMHHIEESFQ
EIKRAIQAKDTFPNVTILSTLETLQIIKPLDVCCVTKNLLAFYVDRVFKDHQEPNPKILRKISSIANSFLYMQKTLRQCQ
EQRQCHCRQEATNATRVIHDNYDQLEVHAAAIKSLGELDVFLAWINKNHEVMFSA
>HMGCR
MLSRLFRMHGLFVASHPWEVIVGTVTLTICMMSMNMFTGNNKICGWNYECPKFEEDVLSSDIIILTITRCIAILYIYFQF
QNLRQLGSKYILGIAGLFTIFSSFVFSTVVIHFLDKELTGLNEALPFFLLLIDLSRASTLAKFALSSNSQDEVRENIARG
MAILGPTFTLDALVECLVIGVGTMSGVRQLEIMCCFGCMSVLANYFVFMTFFPACVSLVLELSRESREGRPIWQLSHFAR
VLEEEENKPNPVTQRVKMIMSLGLVLVHAHSRWIADPSPQNSTADTSKVSLGLDENVSKRIEPSVSLWQFYLSKMISMDI
EQVITLSLALLLAVKYIFFEQTETESTLSLKNPITSPVVTQKKVPDNCCRREPMLVRNNQKCDSVEEETGINRERKVEVI
KPLVAETDTPNRATFVVGNSSLLDTSSVLVTQEPEIELPREPRPNEECLQILGNAEKGAKFLSDAEIIQLVNAKHIPAYK
LETLMETHERGVSIRRQLLSKKLSEPSSLQYLPYRDYNYSLVMGACCENVIGYMPIPVGVAGPLCLDEKEFQVPMATTEG
CLVASTNRGCRAIGLGGGASSRVLADGMTRGPVVRLPRACDSAEVKAWLETSEGFAVIKEAFDSTSRFARLQKLHTSIAG
RNLYIRFQSRSGDAMGMNMISKGTEKALSKLHEYFPEMQILAVSGNYCTDKKPAAINWIEGRGKSVVCEAVIPAKVVREV
LKTTTEAMIEVNINKNLVGSAMAGSIGGYNAHAANIVTAIYIACGQDAAQNVGSSNCITLMEASGPTNEDLYISCTMPSI
EIGTVGGGTNLLPQQACLQMLGVQGACKDNPGENARQLARIVCGTVMAGELSLMAALAAGHLVKSHMIHNRSKINLQDL
Q
GACTKKTA

>TFPI

MIYTMKKVHALWASVCLLLNLAPAPLNADSEEDEEHTIITDTELPPLKLMHSFCAFKADDGPCKAIMKRFFFNIFTRQCE
EFIYGGCEGNQNRFESLEECKKMCTRDNANRIIKTTLQQEKPDFCFLEEDPGICRGYITRYFYNNQTKQCERFKYGGCLG
NMNNFETLEECKNICEDGPNGFQVDNYGTQLNAVNNSLTPQSTKVPSLFEFHGPSWCLTPADRGLCRANENRFYYNSVIG
KCRPFKYSGCGGNENNFTSKQECLRACKKGFIQRISKGGLIKTKRKRKKQRVKIAYEEIFVKNM

>IL6

MNSFSTSAFGPVAFSLGLLLVLPAAFPAPVPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCES
SKEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQNRFESSEEQARAVQMSTKVLIQFLQKKAKN
LDAITTPDPTTNASLLTKLQAQNQWLQDMTTHLILRSFKEFLQSSLRALRQM

>BST1

MAAQGCAASRLLQLLLQLLLLLLLLAAGGARARWRGEGTSAHLRDIFLGRCAEYRALLSPEQRNKNCTAIWEAFKVALDK
DPCSVLPSDYDLFINLSRHSIPRDKSLFWENSHLLVNSFADNTRRFMPLSDVLYGRVADFLSWCRQKNDSGLDYQSCPTS
EDCENNPVDSFWKRASIQYSKDSSGVIHVMLNGSEPTGAYPIKGFFADYEIPNLQKEKITRIEIWVMHEIGGPNVESCGE
GSMKVLEKRLKDMGFQYSCINDYRPVKLLQCVDHSTHPDCALKSAAAATQRKAPSLYTEQRAGLIIPLFLVLASRTQL